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# Newborn Screening by Tandem Mass Spectrometry: Impacts, Implications and Perspectives

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## 1. Introduction

There are over 6000 inherited human diseases described in the McKusick Online Mendelian Inheritance in Man database (OMIM)(McKusick-Nathans Institute of Genetic Medicine, 2011). They show a wide range of variability in frequency, severity, age of onset, diagnostic and treatment approaches. A subset of diseases induce severe metabolic disturbances in the newborn that can lead to irreversible damage and illnesses. For some of those, there is a treatment that prevents such damages as long as it is initiated early after birth. For these diseases, population-based newborn screening has been proposed, developed and implemented in many countries albeit at different rates and based on different criteria. Even if those diseases are rare, the burden that is prevented if treated early has been shown to be cost-effective as it is larger than the cost of systematic screening and treatment of the cases identified (Venditti et al., 2003). Different analytical methods have been deployed since the 1960's to detect abnormal levels of specific metabolites or hormones in the newborn's blood with sufficient reliability and low cost to allow their use as screening methods (see 1.1).

We will describe the tremendous impact that tandem mass spectrometry has had in the field of newborn screening for inherited diseases in the recent decade. After a brief history of newborn screening, we will describe the classical WHO criteria for population-based screening for disease, summarize impacts of tandem mass spectrometry in this field. Then, we will describe the laboratory workflow, the pre-examination, examination, and post-examination aspects of MS/MS-based newborn screening, its advantages and limitations. Other issues will also be reviewed including sample and data management, revision of screening criteria and future perspectives for MS/MS in population-based screening.

### 1.1 History of newborn screening

The concept of universal population-based newborn screening (NBS) for inherited metabolic diseases was introduced in 1963 when a bacterial inhibition assay for phenylalanine in blood spots collected from newborn babies was proposed (Guthrie & Susi, 1963). This systematic screening identified, within the first days of life, babies with phenylketonuria (PKU). PKU, an autosomal recessive inborn error of metabolism is due to the deficiency of phenylalanine

hydroxylase (a cellular enzyme) that leads to the accumulation of phenylalanine in the tissues and blood of affected individuals (20-fold levels as compared to normal) (Scriver, 2007). Phenylalanine at such levels is neurotoxic and impairs the normal development of brain functions after birth, amongst other problems. It affects about one newborn out of 10,000 births in populations of Caucasian origins (1:4500 to 1:18000) (DiLella et al., 1986). If left undetected and untreated, PKU leads to irreversible mental retardation which can be prevented with a low phenylalanine diet initiated rapidly at birth. Newborn screening of PKU has become the classical example of a good screening practice and is accepted as a standard care in all developed countries (Lehotay et al., 2011).

In the following decade, only a few other diseases have been added to NBS, especially congenital hypothyroidism (CH). CH has an estimated incidence of 1:200 to 1:4000 (Rastogi & LaFranchi, 2010). Initially thyroxine (a lipophilic small molecule hormone) was measured as the screening entry test but this was later replaced with measurements of TSH (a peptide hormone) by immunoassay methods. It is estimated that approximately 25% of all newborns worldwide are tested at least for hypothyroidism (Wilcken, 2007). Depending on the incidence of other specific detectable and treatable metabolic diseases some countries have added a few more diseases (e.g. galactosemia, cystic fibrosis (CF), congenital adrenal hyperplasia (CAH) and, tyrosinemia) were added in certain countries (Pitt, 2010).

In 1990, it was proposed that MS/MS could be used to test for multiple analytes simultaneously in dried blood spots from newborn screening programmes (Millington et al., 1990). However, at that time, a significant technical challenge constituted in analyzing hundreds of samples per day injected individually by means of a probe that needed to be cleaned before the next sample (Pollitt, 2006). This laborious process was relieved by the application of electrospray ionization to biomolecules and availability of commercially developed robust and easily cleaned sources of ions, as well as data systems to manage the resultant output (Pollitt, 2006; Johnson et al., 1996; Rashed et al., 1995). The following years revealed that MS/MS, which was already used in the clinical laboratory for quantitation of metabolites and drugs, could also be used as a primary method for NBS of inborn errors of metabolism. This technology was suitable because most of the molecules to measure were small metabolites, it allowed simultaneous analysis of a wide variety of different analytes, it was compatible with high-throughput (2-3 min per sample) and low reagent costs at the same time as being sensitive and specific (Chace, 2009). It has been estimated that, when using the American College of Medical Genetics (ACMG) panel of markers (see below), one affected newborn will be identified by MS/MS for every 3000 births (Rinaldo et al., 2006).

Year	Milestone	Reference
1963	PKU Screening (bacterial growth inhibition)	(Guthrie & Susi, 1963)
1968	WHO Screening Criteria	(Wilson & Junger, 1968)
1973	Addition of Congenital Hypothyroidism Screening	(Dussault & Laberge, 1973)
1975	Tyrosinemia Screening in specific population (Québec)	(Laberge et al., 1975)
1990	Proposal to use MS/MS for newborn screening	(Millington et al., 1990)
1993	Proof of concept for NBS by MS/MS	(Chace et al., 1993)
1995	Electrospray Ionization applied to NBS	(Rashed et al., 1995)
2006	ACMG proposed panel for NBS by MS/MS	(ACMG Newborn Screening Expert Group, 2006)
2008	Revised newborn screening criteria	(Andermann et al., 2008)

Table 1. Some of the major milestones for newborn screening.

## 1.2 Criteria for population screening

Population screening traditionally has aimed only at diseases that fulfill a set of widely accepted criteria for population-based screening proposed in 1968 by Wilson and Junger, as commissioned by the World Health Organization (WHO) (Wilson & Junger, 1968; Table 2).

Principal Wilson and Jungner Criteria for Screening (WHO, 1968)	In short
<ul style="list-style-type: none"> <li>• Knowledge of disorder and its natural history if screening did not occur;</li> <li>• Availability of, access to and knowledge of how presymptomatic interventions change natural history of disorder, prevented irreversible harms, improved outcomes and reduced morbidity and mortality;</li> <li>• Screening test is simple with very high sensitivity and specificity and cost benefit ratio;</li> <li>• Resources exist for follow up of individuals with positive screening for confirmatory testing, introduction and monitoring of treatment and for counseling of family in the case of genetic diseases;</li> <li>• These Principles are acceptable to the consumer, the “target population”.</li> </ul>	<ul style="list-style-type: none"> <li>• <b>Knowledge of disorder</b></li> <li>• <b>Presymptomatic intervention improving outcomes</b></li> <li>• <b>Simple and low cost test with excellent performances</b></li> <li>• <b>Resources available for follow-up of positive individuals</b></li> <li>• <b>Acceptability of program</b></li> </ul>

Table 2. Principal Wilson and Jungner criteria for screening.

These criteria are applicable to systematic or population-based screening (i.e. of asymptomatic persons) for any type of disease and have been considered best practice since their publication in 1968. They thus apply not only to inherited diseases but to screening for any disease. They have been used by public health authorities to evaluate candidate tests or programs for population based screening. Since only few tests and diseases have historically met the criteria, population-based screening programs, including newborn screening, have historically targeted very few diseases. The first criteria asks that the natural history of the disorder be very well known, especially if there is no screening offered. This includes the proportion of untreated individuals that will develop severe and irreversible organ damages before they develop symptoms and seek a medical consultation. That is, because population-based screening (pre-symptomatic by definition) will usually not be cost-effective if the progressing disease causes symptoms before producing severe consequences. The second criteria refers to the presence of evidence that, if the disease is detected before it generates symptoms, there is an intervention (or treatment) to propose that will improve the outcomes. If there is no effective intervention with proven beneficial effects that can be offered to prevent the development of the disease after a positive screening test, then there may be no point in performing this test. The third criteria asks for a screening test that is simple, costs little and has good clinical specificity and sensitivity. In a screening context, sensitivity is very important as the very purpose of a screening program is to detect most if not all individuals that will develop the disease, thus the false negative rate (the proportion of disease cases that will test negative) must be as low as possible. However, if the screening

test has poor specificity and too many false positive results (normal individuals that will test positive), then too many positive results will need to be confirmed by further testing which will not be cost-effective at all. Indeed, one of the major cost drivers for a screening program is the rate of false positive screening results. This is easily understood, as for example, if a disease has an incidence of 1 in 10000 newborns, and the false positive rate is 5%, then for each true positive case, the screening test will produce 500 false positives that will need to be reclassified as negative after a confirmation test. The fourth criteria refers to the availability of health care and financial resources to provide the true positive cases with appropriate follow-up and treatment, as well as the necessary genetic counseling if the disease screened is an inherited condition. Finally, any population-based screening program also needs to be acceptable to the population to whom it will be offered. This is also an important aspect as social acceptability of a program can significantly affect participation rate and undermine the very objectives of a screening program.

With the advent of multiplex screening methods such as MS/MS, allowing simultaneous detection of several tens of analytes in a single run, these criteria have been challenged and it has been proposed to revise them (Section 4) (Andermann et al., 2008). Countries and health care jurisdictions are still attempting to find a balance between the technological drive to provide all the data available when analyzing a newborn sample by MS/MS and the necessity to fulfill rational criteria before screening entire populations for diseases.

### **1.3 Impact of MS/MS spectrometry on newborn screening**

Tandem mass spectrometry, by its very nature, has the potential to detect and quantify many molecules of similar physicochemical properties at the same time, provided the appropriate sample preparation steps and technical parameters are used (see below). This constitutes a dramatic change as compared to the classical methods used for newborn screening before the advent of MS/MS in this field. This is the major reason for the accepted fact that MS/MS has profoundly changed the paradigm of newborn screening (Chace et al., 2003), and possibly of screening per se. The wide variety of newborn screening panels that are currently in operation in various countries (Table 3) exemplifies the choices that decision makers in each health jurisdiction have to make especially with respect to which diseases to screen for (or not). Not all countries have published the results of their newborn screening programmes but it is interesting to see that the incidence of infants with inherited metabolic disorders identified by MS/MS screening is significant, ranging from 1:1800 to 1:6200 newborn. Also, according to the information publicly available at the present time, the number of metabolic disorders screened by MS/MS in newborns ranges from 0 to over 40. Many jurisdictions have not implemented MS/MS yet, while others are still refining their panel of diseases that are included in the screening assays. The reasons for this wide variety of practices include accessibility to funding for an expensive clinical MS/MS screening infrastructure, but also the absence of uniformly accepted practice guidelines with respect to what diseases should be included in a newborn screening programme. While the American College of Medical Geneticists (ACMG Newborn Screening Expert Group, 2006) recommended a large core panel of 29 inherited metabolic diseases, the UK National screening committee (UK National Screening Committee, 2011) recommends screening for only five diseases (Watson et al., 2006). It is worth mentioning here that, while MS/MS is a powerful diagnostic and measurement technology, some of the analytes to be measured in NBS are not easily amenable to MS/MS (e.g. hemoglobins and thyroid hormones).

Country/ Province/ State	Total number of diseases screened for	Number of diseases screened by MS/MS	Overall prevalence of infants with inherited metabolic disorders identified by MS/MS	Reference
Canada (Québec)	4	1	nd	(Laflamme et al., 2005)
Canada (Ontario)	25	20	nd	(Children's Hospital of Eastern Ontario, 2011)
Canada (Saskatchewan)	32	nd	nd	(Lehotay et al., 2011)
USA (New York)	47	37	nd	(Wadsworth Center, 2011)
USA (Minnesota)	30	29	1:1816	(Rinaldo et al., 2006)
USA (California)	50	44	nd	(California Department of Public Health, 2011)
UK (recomm.)	5	nd	nd	(UK National Screening Committee, 2011)
Australia (Victoria)	25	23	nd	(Victorian Government Department of Health, 2011)
Spain	nd	10	nd	(Bodamer et al., 2007)
Switzerland	2	0	nd	(Bodamer et al., 2007)
Austria	nd	25	1:2855	(Kasper et al., 2010)
Taiwan	nd	21	1:6219	(Niu et al., 2010)

Table 3. Comparison of some existing NBS programs worldwide (nd=no data).

## 2. Laboratory considerations

### 2.1 Laboratory workflow and management

A typical newborn screening laboratory receives between 100 to 1000 dried newborn blood spot samples (See 2.2.1) per working day for analysis. The general target for the turn around time to perform the analytical screening procedure and producing the laboratory report is two to four working days, because for some diseases treatment must be started rapidly to prevent irreversible damage to the infant (See 2.4.2). Confirmatory analyses are needed rapidly after a positive screening test result. Thus, although the same tests are repeated, there are several levels of complexity and management issues to address due to the sheer volume and time pressure facing the NBS laboratory. With such constraints, the NBS laboratory needs to prevent any type of service interruption and have back-up solutions to cover all possible aspects of the process with appropriate redundancy. Also, the clinical newborn screening laboratory should implement a Quality Management System for all laboratory processes as proposed in best practice guidelines such as ISO15189 (International Organization for Standardization, 2003) and NCCLS GP26 (NCCLS, 2004). This must be in place to ensure real-time measurement of appropriate indicators of the system's integrity in all phases of the pre-examination, examination and post-examination processes.

Apart from the laboratory processes themselves (see below), the NBS laboratory will usually be at the center of a complex set of activities surrounding the NBS programme. It is not in the scope of this chapter to describe those aspects in details but here are some examples. There are processes that precede the sampling procedure and for which the NBS laboratory

may be responsible, such as managing and maintaining the stocks of virgin sampling cards and forms in the various hospitals and nurseries participating in the program. Systematic follow-up processes should be in place to ensure that no sample is left unattended and to rapidly refer positive cases to a health care facility that will initiate treatment very rapidly. Laboratory managers may also be in direct contact with the public agency commissioning the NBS programme, but also with health care facilities and professionals upstream and downstream of the sampling-to-reporting process itself. There is wide variability in the organization of the different NBS programmes worldwide.

## NBS Laboratory Flowchart

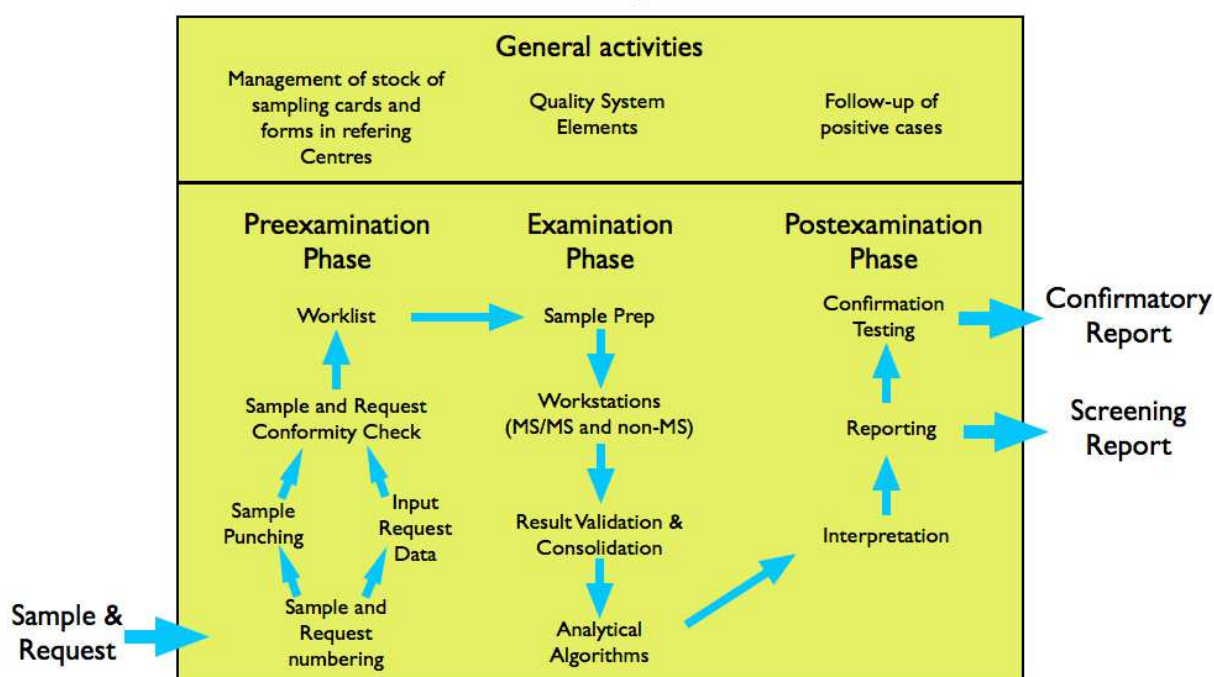


Fig. 1. NBS Laboratory Flowchart.

With respect to the NBS laboratory activities per se (see Figure 1) the three classical pre-examination (or preanalytical), examination and post-examination (or postanalytical) phases include the various stages and processes involved in sample and request forms flows between their arrival in the laboratory and the reporting of results. Data management is described later in this chapter and the details of each phase through which samples and forms go through are also discussed in the following sections.

### 2.2 Pre-examination considerations

Pre-examination conditions are crucial in order to ensure the performance of specimen analysis. The time of the sampling, the delay between the sampling and reception in the laboratory, the baby's condition (e.g. prematurity, parenteral nutrition, recent transfusion) can influence screening results and thus the life of the baby. Due to these limitations, it is important to note that the screening test result is not diagnostic and requires confirmation with an independent sample and method (Pitt, 2010). It has been estimated that there is a measurement error of  $\pm 30\%$  due to the fact of using blood dots on filter paper as sample in part because it is done in many locations and by many people (Lehotay et al., 2011). Of note,

with metabolic diseases, several-fold increases of metabolite levels are usually expected. In addition to specimen collection, storage and transport are also potential sources of errors.

### 2.2.1 Dried blood spots as samples and their management

#### Specimen collection

Collection of blood onto an absorbent paper card (dried blood spots: DBS) represents the most common type of NBS sample. On a filter paper, a few drops from a heel prick are dried in air for a few hours before being sent to the NBS laboratory. This filter paper is attached with a card that includes specific spaces for writing the family names of the father, the mother, their address and phone number, the date and hour of the newborn’s birth, the date and hour of sampling, and the birth hospital (Figure 2). Each NBS card has a unique number or bar code. In the province of Québec, a multiple identification of the newborn is done by 1) names of the parents, 2) the sampling card number which is unique for the each newborn and provides information of the location of the birth and 3) the date of birth. A complete information card is mandatory for the specimen to be valid and accepted by the NBS laboratory. Otherwise, a new and appropriately identified sample is needed.

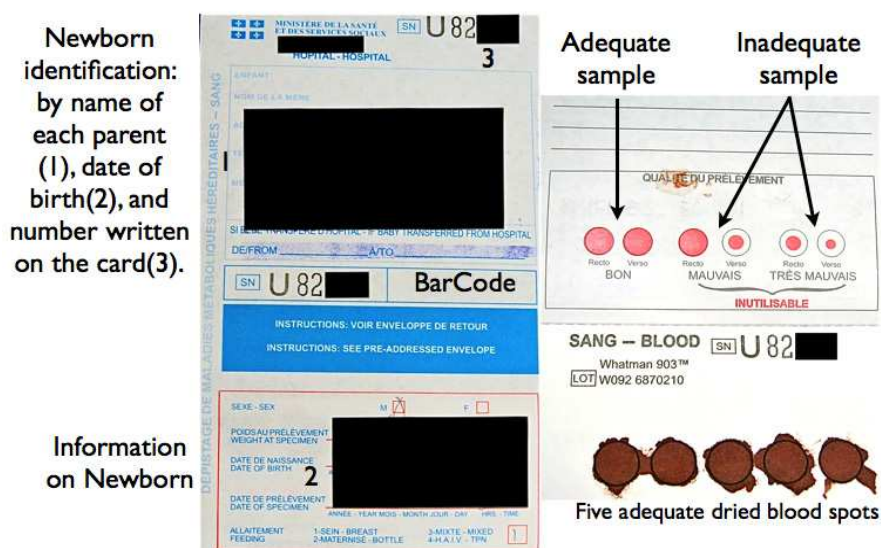


Fig. 2. Example of a newborn screening blood sampling card and its attached filter paper.

The heel puncture must be done on the planar surface of the foot in the safest area (medial to a line drawn posteriorly from the middle of the great toe to the heel or lateral to a line drawn from between the 4<sup>th</sup> and 5<sup>th</sup> toe to the heel.) according to the H3A6 CLSI guidelines. No foreign substance around the filter paper must contaminate the sample. Blood must completely fill all printed circles and is applied evenly on one side of the filter (but must also fill completely the minimal sample circle on the other side of the paper), free of layering and clots. It must be correctly dried (4 hours of air drying at room temperature). Laboratory analysis of the sample depends on an assumed amount of blood in the filter paper. Criteria for adequate sampling are summarized by Wadsworth Center (2011) and Kayton (2007). Timing of collection is important because some metabolite’s levels vary with the infant’s age (Wilcken & Wiley, 2008). Sampling within 24-72 hours of age is common practice. If it occurs too early (<24 hours of age), a new specimen may be required. Prematurity, birth weight,



parenteral nutrition, transfusion, type of feeding, neonatal jaundice and drugs affect metabolite levels (Rolinski et al., 2000) and may influence laboratory results. Such facts should be documented on the NBS card to help interpretation of results (Pitt, 2010).

### **Transport**

Delays in transport can cause degradation of some markers and result in false negative results. Samples should be sent within 24 hours after collection. Batching causes delays in reception and processing of samples and may have serious consequences on the life of an affected newborn (Kayton, 2007). Upon arrival of samples to the NBS laboratory, verification of sampling card information and validation of specimen adequacy is performed. A laboratory identification number is generated for each sample card.

### **2.2.2 Automation of pre-analytical steps**

Given the large volume of samples to analyze in a timely and reproducible way, automation of some pre-analytical steps has been implemented in many NBS laboratories. A micro-volume pipetting station and punchers help to shorten turn around time in the NBS laboratory. The puncher (basic, semi-automatic or automatic) is a convenient tool with minimal user intervention. It punches the sampling cards where a blood spot is located and drops the punch into a microplate. Punchers can read bar codes and some have a light guide to help the technician to optimally position the sample card and verify that the disk falls into the well. More sophisticated punchers are fully automated and accept 300 samples at a time. The micro-volume pipettor can pipet 96 well plates. It is a compact unit, with high precision (1µl /min dispensing, CV 5%) control of dispense rate and calibration values.

## **2.3 Examination considerations**

In the following section, we discuss the particular aspects of tandem mass spectrometry as applied to the analysis of dried blood spot samples for newborn screening (NBS).

### **2.3.1 Types of sample preparation procedures**

In the context of NBS, two types of pre-analytical sample treatments are in use: with or without derivatisation. After reception, verification and labeling of NBS sampling cards, a punch (generally 3.2 mm in diameter) is deposited in a 96 well plate and the position of each specific sample is noted (usually electronically by reading a bar code on the sampling card). Extraction of the analytes from sample card punches is usually performed with a solution comprised mainly of methanol, but including isotope-labeled internal standards for the main amino acids and acylcarnitines that will be measured. Quantification of the metabolites of interest will use these internal standards as a reference. The derivatisation method is more frequently used because of its greater sensitivity. This method will produce butyl derivatives. New methods without derivatisation are becoming more popular with the help of new generations of MS/MS instruments with increased sensitivity. This method simplifies the sample preparation steps by removing the butylation of metabolites with a toxic and corrosive reagent. This reduces significantly sample preparation time. For the majority of quantitative results for acylcarnitines and amino acids, the two methods differ by less than 15% (De Jesus et al., 2010). However, larger differences are observed for dicarboxylic acylcarnitine compounds, especially for short chains such as C5DC (glutaryl-carnitine). Raw counts for the non-derivatised method are much smaller as compared to the derivatisation method, although quantitative results are similar. C5DC is a major marker for

glutaric aciduria type I. It is thus important to pay particular attention to this marker. The newer and more sensitive generation of MS/MS instruments may compensate for these lower values. Use of a non-derivatised method can induce a lack of differentiation of isobaric acylcarnitines, as well as increase false-positive rates for certain disorders, and cut-offs should be adjusted accordingly (De Jesus et al., 2010).

### 2.3.2 Technical configurations of MS/MS for newborn screening

A typical configuration for tandem mass spectrometry for a newborn screening laboratory includes four components (Figure 3). The first one is an HPLC-type of pump ensuring the flow of a mobile phase, but with no liquid chromatography step. The second component injects samples into the mobile phase before it reaches the MS/MS. Pipetting of each sample is performed by an auto-sampler for 96-well plates and hundreds of samples per day.

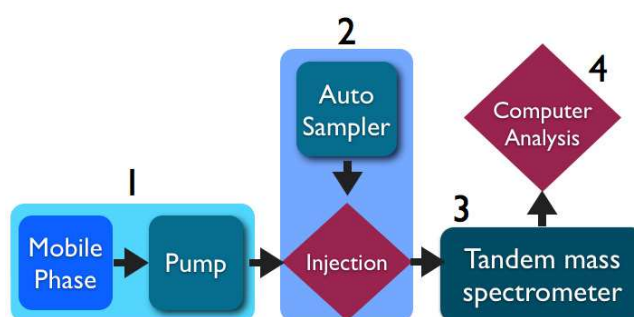


Fig. 3. Schematic configuration for tandem mass spectrometry for newborn screening.

The third component comprises the two mass spectrometers that scan the analytes in each sample, identify and quantify the various signals from metabolites of interest and transfer the raw information to the fourth component, a computer system, which performs the formal quantification of each metabolite and integrate the information into a useful data set.

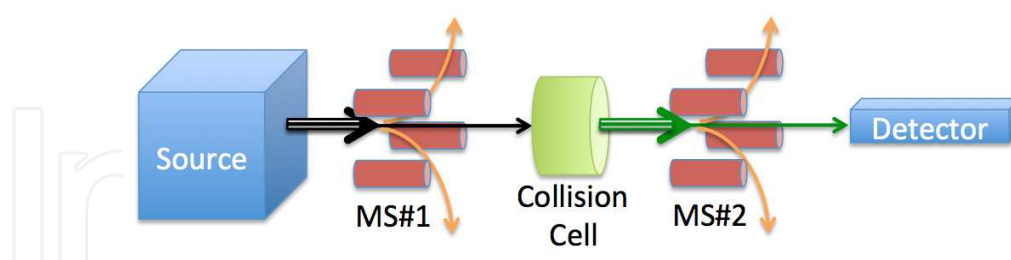


Fig. 4. Schematic representation of the flow of ions in a tandem mass spectrometer. Adapted from Waters Micromass Quattro Premier XE Mass Spectrometer Operator's Guide.

The tandem mass spectrometer itself comprises five major elements (Figure 4) : 1- the source where samples in liquid phase are injected, ionized and introduced into the first quadrupole MS; 2- the first quadrupole MS, where a first selection of ions by their  $m/z$  ratio is performed (many ions with similar  $m/z$  ratios may be selected); 3- the beam is directed into a collision cell where ions collide with Argon molecules which break ions having entered the cell (mother ions) into smaller ions (daughter ions) specific to each mother ion; 4- the second quadrupole MS receives these fragments and filters the daughter ions of interest (green arrow) to identify the presence of molecules of interest, with internal standards to

support quantification; 5- an ion detector counts each ion that passes through the second MS at each specific  $m/z$  ratio and transfers the information to the computer system for analysis. Many types of sources of ions are used in mass spectrometry, depending on the application. An important aspect of tandem mass spectrometry applied to NBS is electrospray ionization (ESI). ESI is a method of obtaining ions without destroying molecules in the sample. It was developed by John Bennett Fenn who received the Nobel prize of chemistry for this in 2002. Before the development of ESI, it was very difficult to directly inject a liquid phase into a MS, as the interface between a liquid chromatograph and the MS was not easily performed; only gas chromatography was easily interfaced with MS. ESI (Figure 5) operates by sending a liquid sample through an electrically charged capillary tube. A continuous flow of nitrogen along the capillary creates a spray of charged liquid at the end of the capillary. Charged droplets are formed and their evaporation shrinks their size until they burst due to electric repulsion, which leaves individual molecules that are charged and ready to be injected into the MS, after being accelerated according to their charge.

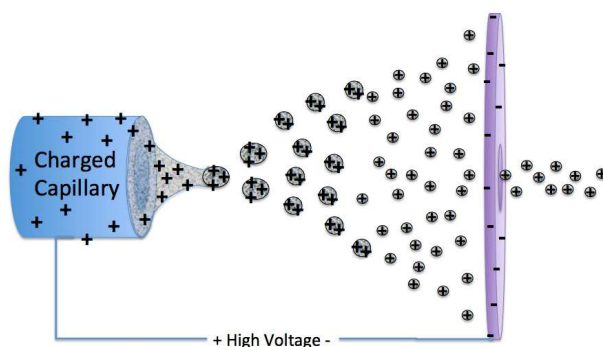


Fig. 5. Schematic representation of electrospray ionization. The liquid sample flows through a charged capillary (left), which produces positively charged droplets attracted by a negatively charged electrode that accelerates charged micro droplets into the MS/MS. *Adapted from Balogh & Stacey (1991).*

Finally, ions are selected and analyzed according to their mass to charge ratio ( $m/z$ ) by the quadrupoles in tandem. Each quadrupole consists in four parallel rods defining the edges of a rectangular cuboid. Each rod has an opposite charge to its neighbor rod and the same as its opposite rod. The value of the  $m/z$  ratio of ions selected by the quadrupole is determined by a radio frequency superimposed with a continuous voltage applied to the quadrupole's electrodes (rods). For a specific radio frequency, there is a cut-off where ions of a specific  $m/z$  and larger will pass through. Thus the radio frequency acts as a filter of minimal  $m/z$  ratio. Inversely, the combination of continuous potential and the radiofrequency act as a filter of maximal  $m/z$  ratio. Thus addition of the appropriate voltage to a radio frequency selects a specific  $m/z$  ratio under which ions will not pass through the quadrupole.

### 2.3.3 Performing the analysis

In the context of newborn screening, the objective of the analysis is to identify selected inherited metabolic disorders of clinical significance.

As for any laboratory analysis, prior to offering a clinical laboratory test, the laboratory must validate (in the case of home-made reagents) or verify (in the case of commercial kits) the analytical method it will be implementing. This will usually include correlation with an

established (or other validated) method, intra-run and inter-run reproducibility studies, carryover studies, linearity, recovery, precision, trueness, etc. These studies are needed to ensure the robustness and performance of the analytical method and the overall analytical process in the specific context of a physical laboratory facility. Cut-offs for detection of metabolites of interest should also be established. Accuracy of the assay is important near the cut-off value for each analyte. During the set-up phase for MS/MS assays in the laboratory, up to 2500 to 4000 samples may be needed to establish the appropriate cut-offs for metabolites levels that will be considered as positive upon screening results. Cut-offs are also compared with other screening laboratories and can be adjusted with the collection and analysis of larger number of samples over time. The difficulty is to establish a cutoff value for each metabolite in order to detect diseases and, at the same time, to minimize the occurrence of false negative and false positive screening results.

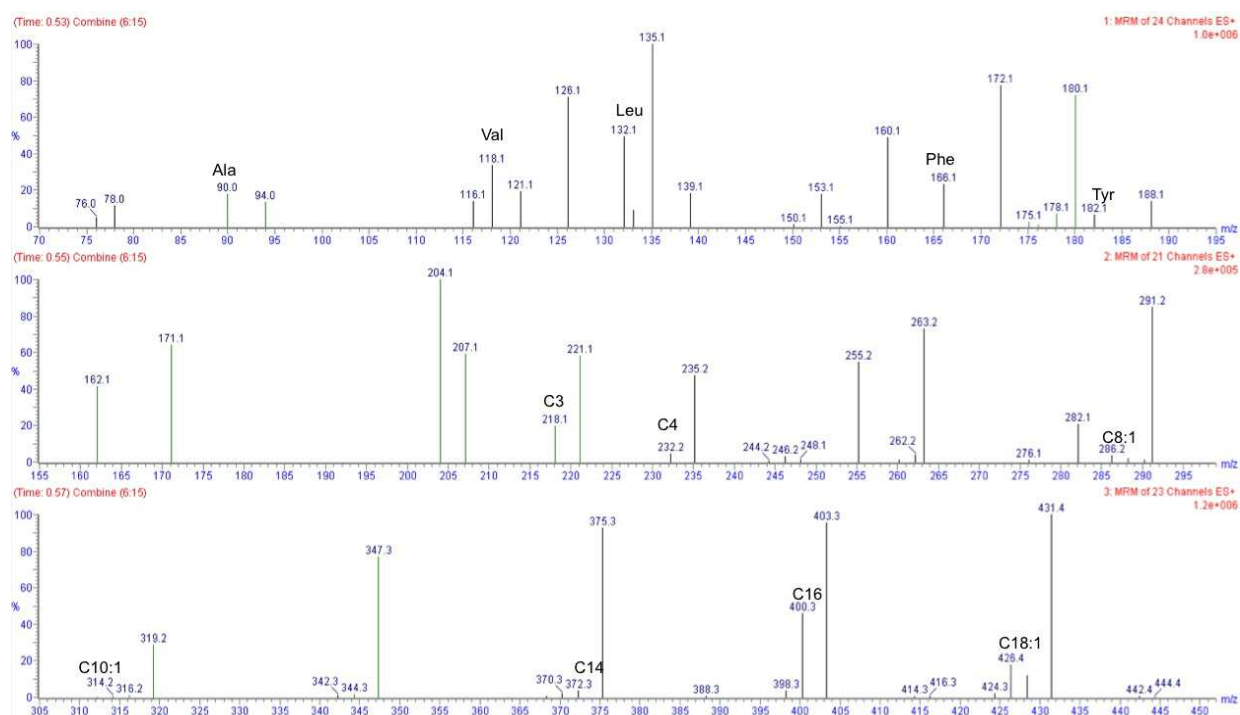


Fig. 6. Typical output for a newborn sample analysed by Multiple Reaction Monitoring (MRM) in MSMS. In this MSMS spectrum, targeted ion masses of interest, corresponding to markers for several inherited metabolic diseases, are divided in three parts: 1: MRM of 24 amino acids; 2: MRM of 21 acyl-carnitines from C0 to C8:1; 3: MRM of 23 acylcarnitines from C10 to C18OH; x axis =  $m/z$ ; y axis = % intensity which relates to each metabolite concentration. Concentrations are compared with cut-offs for each corresponding analyte, defining an abnormal or normal metabolic profile for this newborn.

Also, prior to launching the test offer, the clinical laboratory must implement a quality assessment program for the analytes that will be measured. A typical output for a newborn sample is shown in Figure 6. Each run is checked by inspection of TIC (total ion current chromatogram) of each sample, internal standard and all quality control (QC) samples. In the laboratory, quality controls (QC) (low and high levels) are run with each batch of newborn samples and contribute to determining the adequacy of the run. Low level controls reflect the normal endogenous metabolites and high level controls reflect concentrations

near the cut-off value or an abnormal state for each analyte. For each plate a minimum of one low QC and one high QC must be present. These QCs are “internal quality controls” given that their expected value is known to the lab performing the analysis. It is advised that other controls (such as external controls (of unknown value) or known cases) would also be present in the plate (Lehotay et al., 2011). With proficiency testing (external QC), there are several different dimensions of the screening processes that are examined. The laboratory processes - as if they were real samples - materials of unknown value (to the testing laboratories) provided by a proficiency testing program that then compares the results from several different participating laboratories. The complexity in developing the QC materials for MS/MS in NBS is high. Moreover, participation and comparison of results within collaborative projects such as Region 4S project (Region 4 Genetics Collaborative, 2007), and/or to a quality control program such as the CDC program where performance criteria are collected from several laboratories is relevant and useful to test the performance of the NBS laboratory and essential to insure short and long term quality of results (Chace et al., 1999; Adam et al., 2000; Hannon et al., 1997).

#### **2.3.4 Advantages and limitations of MS/MS**

MS/MS technology offers a new approach to NBS by having the ability to screen rapidly for 30 or more metabolic disorders in a single analysis from one small blood sample using a simple protocol (Chace et al., 2003). Indeed, in one run, more than 40 analytes can be quantified. This capacity decreases the NBS laboratory turn around time because many metabolites can be measured in parallel as opposed to sequentially. It is also potentially clinically efficient because screening of metabolic disorders asks for more than one marker to be measured to define the disease. With MS/MS, there is the possibility to produce a rather complete metabolic profile for a patient in a single run. However this great advantage comes with the limitation that some “disorders” detected by MS/MS can be benign or mild. An ethical question is thus raised with respect to how the laboratory, and the physicians, will deal with this information that is of undetermined clinical value (Lehotay et al., 2011). As compared with other methods used in the past, MS/MS showed a high analytical performance for detecting diseases of high frequency, notably MCAD deficiency and PKU. However, the technology’s analytical performance is not identical for all analytes measured. (for instance succinyl acetone for the screening of tyrosinemia Type I). Thus, some important diseases of the newborn such as tyrosinemia type I have higher false positive rates by MS/MS than by more conventional methods (Chace et al., 2003). This led to the development of a number of second tier tests requiring a separate testing protocol with a rapid turn around time (Magera et al., 2006; Sander et al., 2006). Another limitation of MS/MS is raised by the diagnostic dilemma when one or several markers are the same for more than one disease. For instance C5OH is a marker of severe disease such as holocarboxylase synthase deficiency as well as a marker for a generally benign condition, namely 3 methylcrotonylCoA Carboxylase deficiency (Dantas et al., 2005; Koeberl et al., 2003). Despite these limitations, implementation of tandem mass spectrometry has generated a significant evolution in newborn screening programs, often referred-to as a paradigm shift.

#### **2.3.5 Metabolites measured**

Several metabolites are measured by MS/MS in a newborn screening laboratory. Generally, they arise from protein metabolism (amino acids), fatty acid and organic acid metabolism (acylcarnitines), or from endocrine metabolism (hormones or specific metabolites). To

determine those metabolites to be integrated to a newborn screening MS/MS profile, it is important to have an excellent understanding of human metabolism. An enzymatic defect along a metabolic pathway can result in accumulation of the substrate and insufficient levels of the product of this enzyme. The accumulation of substrate - or its by-products -, or the decreased concentration of a normal product - or by-product - can be identified in the MS/MS spectrum and quantified. One challenge in newborn screening testing is that some metabolites are markers for several diseases, that is, they are not specific to a single metabolic disorder. To circumvent the issue of disease specificity, ratios of some markers are usually computed (Region 4 Genetics Collaborative, 2007). Extensive lists of markers that can be analyzed by MS/MS for newborn screening profiles are already published (ACMG, 2006; De Jesus et al., 2010; Lehotay et al., 2011). Some diseases that need to be screened for are not yet readily amenable to MS/MS and thus most NBS laboratories also run other types of assays for these conditions.

Because of pre-examination and examination concerns, screening assays are not equivalent to diagnostic assays. In order to allow for high throughput and multiple metabolite detection, some compromises have to be made on the assay's analytical performances. For [false] positive samples, these are in part compensated by more specific 2<sup>nd</sup>-tier assays (see 2.4.1). However, negative screening results will not be retested and thus false negative screening results have to be minimized in the context of a screening program, even if somewhat more false positive results will be generated at the initial screening step.

## **2.4 Post-examination considerations**

After the screening examination phase is completed, the laboratory needs to determine which samples need confirmation and also to produce a laboratory report. As the disorders screened in NBS are rare, the majority of results are normal, but test performance will strongly influence delivery of ambiguous or false positive results, with its implications in terms of repeat testing, investigation, and parental stress. The expansion of NBS to a significantly increased number of disorders by MS/MS technology has only amplified and complexified the management of NBS programs during and following sample analysis.

### **2.4.1 Interpretation of screening results**

If the expansion of NBS by MS/MS is a breakthrough in the field, it also represents a major challenge for NBS laboratory personnel who are faced with a significant increase in data generated, as was mentioned in previous sections (see 2.3.3 and 2.3.4).

Performance characteristics of the NBS procedures will depend upon chosen tests cut-offs. If the threshold level for a disorder is too high, a proportion of newborns with a disorder will be missed, while a threshold that is too low will generate many additional positive screen in infants without the disease. The increased number of conditions tested, each of them being rare, led to the implementation of 2<sup>nd</sup> tier confirmatory tests that are more complex, time-consuming and unsuitable for high throughput mass screening but with performance characteristics of increased sensitivity and specificity. The main aim is to diminish the number of false positive screens. The problem is more acute for primary tests that lack specificity and generate a high rate of false positives (for specific examples, see section 2.4.4). The false positive rate is the proportion of positive tests in newborns that eventually turn out to be normal after follow up evaluation, while the positive predictive value of a test, a function of the sensitivity, the specificity and the prevalence of the condition, is the

probability that the infant tested positive has the disease. With about four millions births annually in the USA and each infant screened for about 30 conditions, assuming a specificity of 99.9% of the primary test for each disorder to be screened, this amounts to about 50,000 false positive results per annum in the USA only.

One must emphasize that the introduction of MS/MS in NBS, a very sensitive and specific technology, is not in itself responsible for the increased number of false positive screens, the phenomenon is rather a consequence of the increased number of extremely rare disorders that are included in the NBS panels. This may be unavoidable if NBS programs aim to screen infants for very rare inborn metabolic disorders. As an example, despite a sensitivity of about 100% and a specificity of 99.96%, after confirmatory testing, only eight (8) newborns were true positives out of the 1249 who initially tested positive for maple syrup urine disease (MSUD) in the USA in 2007 (3,364,612 were tested for MSUD overall) (Schulze et al., 2003). Undoubtedly, NBS protocols must include the diagnostic confirmation of a positive screen before any clinical intervention is instituted, otherwise a large number of infants would be given inappropriate treatment. Targets of adequate analytical and post-analytical performance in the era of NBS expansion by MS/MS multiplex testing have been proposed: false positive rate < 0.3%, positive predictive value > 20% (Rinaldo et al., 2006). Interestingly, novel NBS algorithms are being proposed, and 2<sup>nd</sup> tier testing, characterized by high sensitivity and specificity may also be used to identify rapidly newborns that would have otherwise been missed (i.e. false negatives) by reducing a primary test cut-off followed by 2<sup>nd</sup> testing on the initial blood spot for the increased number of abnormal results from the primary test, enhancing the performance of NBS (Turgeon et al., 2010).

Example of conditions	Primary markers	Example of informative Ratios	Some Other Conditions with same markers
Argininosuccinic acidemia	citrullin	Citrullin/arginin	Citrullinemia
Citrullinemia types 1 and 2	citrullin	Citrullin/arginin	Citrullinemia
Maple syrup disease	Valine, leucine- isoleucine	Valine/phenylalanine Leu-isoleucine/alanine	ketosis
Phenylketonuria	phenylalanine	Phenylalanine/tyrosine	Diet, prematurity
Type 1 Tyrosinemia	Succinylacetone (the best), tyrosine	Tyrosine/citrulline	Prematurity, types 2-3 tyrosinemias
Types 2 and 3 tyrosinemia	tyrosine	Tyrosine/citrulline	Prematurity, type1-tyrosinemias
Medium chain acyl-coA dehydrogenase deficiency	C6, C8, C10:1, C10	C8/C2; C8/C10	Glutaric type 2
Short chain acyl-coA dehydrogenase deficiency	C4	C4/C2; C4/C3, C4/C8	Glutaric type 2
Glutaric acidemia type1	C5DC	C5DC/C5OH, C5DC/C8, C5DC/C16	Glutaric type 2
Propionic acidemia	C3	C3/C2,C3/C16	Cobalamine deficiency (C, D)
Methylmalonic acidemia (Cobalamin deficiency)	C3	C3/C2,C3/C16, C3/Methionine	Propionic acidemia, Cobalamin deficiency A, B

Table 5. Examples of markers for some conditions, adapted from Lehotay et al. (2011).

Thus, not all abnormal results indicate the presence of an inborn error of metabolism and consequently they must be confirmed by a diagnostic or confirmatory procedure (see 2.4.3). Of note, interpretation of results must distinguish between an ongoing pathologic process and changes in metabolite concentrations due to the maturation process of organ systems. Discrimination between a frankly abnormal result and an undetermined result usually rely on the observed pattern of biochemical abnormality. Most inborn errors of metabolism have specific primary analytes associated with the condition (Table 5).

The presence of secondary markers or analyte ratios can increase the specificity of the screening procedure for the disorder associated with a primary analyte. Other concerns also arise in the presence of a positive screen or ambiguous result. Indeed, some conditions, such as prematurity (delivery < 37 weeks gestation), low birth weight (< 2500 g), some medications, total parenteral nutrition and newborn sickness unrelated to an inborn error of metabolism may cause up to 40% of false positive results (Lehotay et al., 2011). In such circumstances, repeat sampling is usually requested. Recently, the CLSI has issued guidelines and recommendations for repeat testing in these specific circumstances (Miller et al., 2009). Reports of abnormal results must thus include a qualitative interpretation by a NBS professional well versed in metabolic patterns associated with disorders, but also with factors that may mimic the presence of a screened condition.

#### **2.4.2 Reporting of results**

Despite recommendations by the American Academy of Pediatrics (AAP) newborn screening task force (Mandl et al., 2002), there exists a wide variety of practices between NBS programs regarding notification of results. While most programs will notify primary health care providers of results from all newborns, whether results are normal or positive, a few states and one Canadian province will issue results only if abnormal. All NBS programs in the USA report abnormal results and monitor follow up activities, but the reporting of abnormal results by NBS programs includes a wide diversity of approaches such as telephone call, letter, certified letter, secured web-based electronic letter and fax. All USA states send a report to the infant's pediatrician, and most report to the hospital of birth, the parents and a geneticist (Kayton, 2007). Considering the importance of follow-up of abnormal results for potentially life threatening conditions, the vast majority of states will follow up until it is confirmed that the screened infant with an abnormal result has a follow up appointment, and about half will track until a diagnosis is made and treatment is initiated. Only a few states however will continue to follow up periodically (Kayton, 2007). Timely notification of parents of newborns screened positive for disorders with potentially disastrous evolution is critical. Notification of results depends on age at blood sample collection (which varies between NBS programs), dispatch, transfer to the NBS laboratory and processing. The UK Newborn Screening Program Center has recommended targets for reception and processing of samples and notification of results using PKU as the prototype disorder (NHS Newborn Bloodspot Screening Programme, 2008). Overall, 99.5% of samples should be received by 16 days of life, samples should be processed within 4 working days, ideally within 2 working days, and 100% of screened positives should be notified before the infant is 18 days old, with treatment initiation by 21 days of life, if confirmed. Ideally, 80% of screened positives should be notified by 12 days of life, with initiation of therapy before 14 days of life of the infant confirmed positive. As discussed previously, the impact of an expanded NBS panel by MS/MS are significant for both the NBS personnel and health care providers: much more interventions and communications to pediatricians are made by the



former, including the reporting of an increased number of false positive and ambiguous results, while the latter are faced with the investigation of infants for disorders for which little is known about the natural history or may be benign conditions. In 2009, the National Academy of Clinical Biochemistry (NACB) issued guidelines for optimal follow up testing for positive newborn screens using MS/MS (Dietzen et al., 2009).

### 2.4.3 Confirmation testing in a NBS program

Classically, a positive NBS result needs a repeat analysis in duplicate from the initial blood spot before repeat sampling is requested. If the result is confirmed positive with the repeated screening test, the need to perform additional testing depends on the disorders for which the infant is presumably positive and the availability of a second tier test for that disorder. Second tier tests are reflex tests performed on the same dried blood sample as the primary screening test. The relevance to perform a 2<sup>nd</sup> tier test is justified by the possibility of false positive screens or the presence of an ambiguous result in the presence of poor specificity of the primary screening test for a specific disorder. As mentioned earlier, the expansion of NBS for multiple rare disorders causes a significant increase in the number of false positive results, which increases the burden on laboratory personnel, health care providers, not to mention parental stress. 2<sup>nd</sup> tier tests allow to confirm results with increased specificity on the initial blood spot. A typical example is NBS screening of congenital adrenal hyperplasia (CAH) using 17-OH progesterone by fluoro-immunoassay. The method lacks specificity, is affected in the presence of prematurity and neonate illnesses, and cross reacts with structurally similar steroids, notably 17-OH pregnenolone. The second tier tests measure 17-OH pregnenolone by LC-MS/MS (Etter et al., 2006), but also cortisol and androstenedione. The ratio of (17 OH-P + androstenedione)/cortisol, increases discrimination since 17-OH-P and androstenedione are increased while cortisol is decreased in CAH (Schwarz et al., 2009; Lacey et al., 2004; Minutti et al., 2004). Another example is the presence of elevated propionylcarnitine (C3), which may suggest the presence of propionic acidemia, but is a non specific marker, also present in methylmalonic acidemia, a disorder of cobalamin metabolism. Further analysis using original blood spots for 3-OH-propionic and methylmalonic acids performed as 2<sup>nd</sup> tier tests allow to rule in or to rule out propionyl and methylmalonyl acidemia. These 2<sup>nd</sup> tier tests increase the specificity of the screening procedure, with less than 5% of positive C3 being confirmed as true positives (la Marca et al., 2007). Follow up testing for plasma acylcarnitines, along with other markers, have been developed to determine if the presumptive screened positive are truly positive and if so, for which condition. A number of other inborn errors of metabolism need a 2<sup>nd</sup> tier test to increase positive predictive value.

### 2.4.4 Short term follow-up of confirmed positive cases

In the presence of a sample that screened positive for a disorder, the NBS laboratory must report the results for rapid clinical evaluation, diagnosis and appropriate management of the newborn. Actions needed for complete and secure transmission of the information are not only dependent upon the NBS laboratory, but must be coordinated by a structured program. Indeed, a number of services will be needed, such as metabolic, enzymatic and/or molecular diagnostic laboratories, but also specialized health care providers such as geneticists, endocrinologists and metabolic dieticians. Newborns screened positive may be referred to either the primary health care providers or directly to tertiary specialized services, depending to the structure of health care services.

Because of the rapid increase in the number of rare disorders screened with the expansion of NBS by MS/MS, primary health care providers are increasingly faced with the referral of infants with a rare disorder. Results may be presented as analytes measured and/or conditions screened, and should include a clear interpretation, especially in the case of screened positive. Importantly, abnormal results of a screened positive do not mean that the infant has the disease, as additional investigation may be needed before a final diagnosis is reached. Classically, treatment is not initiated before the diagnosis is confirmed and parents have received appropriate counseling. With the increased number of conditions screened, it is thus even more imperative that the reporting of screened positive infant be presented in adequate and comprehensible format to the health care provider. To help physicians structure their approach in the provision of care for these newborn, the American College of Medical Genetics (ACMG) has developed a tool, called the ACT Sheet and confirmatory diagnosis, which provides information for each condition involved in NBS. The tool includes 1) a 1-page ACTION (ACT) sheet that describes short term actions and communication with the family and determining the appropriate follow up steps for the infant that has screened positive, and 2) a 1-page algorithm with an overview of the basic steps involved in determining the final diagnosis in the infant (ACMG, 2011). The ACT sheet and confirmatory algorithm exist for various disorders (endocrine, haematological, genetic and metabolic), and each ACT sheet includes links to informational resources.

There is no doubt that expansion of NBS by MS/MS to a large number of disorders poses organisational challenges. As many rare conditions are screened for, NBS programs and health care professional must deal with the uncertainty about the natural history of these rare conditions. The laboratory is central to the whole NBS process: it must develop a test menu that includes specific 2<sup>nd</sup> tier testing protocols in order to confirm the biochemical abnormality and identify the underlying metabolic condition (see Table 6 for examples).

Disorder	Example of follow-up marker(s)	Example of follow-up method(s)
<b>Phenylketonuria</b>	phenylalanine, tyrosine in plasma	ion exchange chromatography (IEC)
<b>MSUD</b>	urine organic acids valine, leucine, isoleucine alloisoleucine in plasma	GCMS IEC
<b>Argininosuccinic acidemia</b>	urine organic acids argininosuccinate, citrulline	GCMS IEC
<b>Medium chain acyl CoA dehydrogenase deficiency (MCAD)</b>	urine organic acid C6, C8, C10 acylcarnitine in plasma	GCMS LCMS/MS
<b>glutaric aciduria type1</b>	urinary organic acid plasma C5DC acyl carnitine	GCMS LCMS/MS
<b>glutaric aciduria type2</b>	urine organic acid plasma C5DC, C5, C5OH, C6, C8, C10-C16 acyl-carnitines	GCMS LCMS/MS
urine organic acid GCMS		
Additional tests with cells and enzymatic assays can confirm the diagnosis		

Table 6. Examples of follow-up testing for positive cases in newborn screening program (adapted from Dietzen *et al.*, 2009).

Highly structured NBS programs will favour the increase in knowledge about these rare conditions, and will ultimately improve provision of care for these infants, communication between parents and health care professional and will reduce parental anxiety related to uncertainty (Deluca et al., 2011). Implementation of electronic health information exchange (HIE) is an opportunity for quality improvement of NBS. With harmonization of standards, coding and terminology and implementation of decision-making and support tools, access to HIE by health care professionals should result in improved effectiveness of short and long term management of infants with an inborn error of metabolism (Downs et al., 2010).

#### **2.4.5 Conservation of dried blood spots**

NBS laboratories receive dried blood spots (DBS) specimens from virtually all newborns from their territory. NBS specimens represent an unbiased source of blood that can generate new population-based knowledge, including potential improved children's health, but also new understanding on the background of both child and adult disease. In addition, NBS is probably the only program reaching the entire population, representing, for instance, about 4 millions births/year in the USA. To ensure proper security, confidentiality, privacy and public confidence in NBS practices, each jurisdiction must regulate NBS practices regarding retention and storage of DBS. Owing to the implementation of molecular techniques using DBS, there is a growing interest in the use of these unique samples for purposes other than NBS, such as genomic research. Stored DBS specimens have also been used to establish the presence of congenital cytomegalovirus infection (Barbi et al., 2006; de Vries et al., 2009) and for forensic purposes (Couzin-Frankel, 2009).

Currently, storage and secondary use of DBS is controversial and two distinct approaches are in use, a short-term storage approach (i.e. < 2-3 years) and a long-term approach (more than 5-10 years). While the first approach allows the standard use of DBS for quality insurance and program evaluation, treatment efficacy, test refinement and result verification, the latter one further allows the use of DBS for research purposes, that is for purposes other than those for which they were originally collected. Parents from Minnesota and Texas have charged their respective health departments because DBS specimens had been stored without parents' knowledge or consent. This is not surprising, in the absence of national guidelines and of diverse practices regarding retention and use of specimen in the USA (Lewis et al., 2011) and Canada (Avard et al., 2006). In Texas, as part of the settlement more than 5 millions DBS specimens dating back to 2002 were destroyed (Grody & Howell, 2010; Rollins, 2011). Recently, the Secretary's Advisory Committee on Heritable Disorders in Newborns Children (SACHDNC) published a briefing paper which reviewed the issues facing the state NBS programs related to retention and use of residual DBS specimens and proposed a foundation for developing national guidelines (Secretary's Advisory Committee on Heritable Disorders in Newborns and Children, 2010). This included recommendations for the implementation of a policy on access, disposition, protection of privacy and confidentiality of residual DBS specimens. Biobanking residual DBS for secondary use was also addressed by the President's Council on Bioethics (President's Council on Bioethics, 2008). Noteworthy, in 1993 the Denmark government implemented a national newborn screening biobank addressing most of these issues (Norgaard-Pedersen & Simonsen, 1999). The Organization for Economic Co-operation and Development (OECD) with the International Society for Biological and Environmental Repositories jointly developed best practice guidelines useful to implement such policies in NBS (Baust, 2008; OECD, 2007).

Use of stored DBS specimens requires stability and integrity of samples. Stability will depend on the analyte to be measured. Non-DNA material such as amino acids left at room temperature may degrade within a few months (Therrell et al., 1996), but may be stable for years at -20°C. Long-term storage at ambient temperature results in significant degradation of acylcarnitines, which are hydrolysed into free carnitine and corresponding fatty acids, and aminoacids (Strnadova et al., 2007). The velocity of the decay is logarithmic, depends on chain length of acylcarnitine (Fingerhut et al., 2009), and appropriate correction for storage should be applied. Acylcarnitines are stable at -18°C for at least one year (Fingerhut et al., 2009). On the other hand, DNA quality from stored DBS specimens at room temperature allowed extraction and successful amplification for at least 25 years (Searles Nielsen et al., 2008). Quantitative RNA stability was also shown from stored residual NBS specimens for 20 years at 4°C in controlled relative humidity maintained at 30% (Gauffin et al., 2009). Specimen-to-specimen contamination should be prevented.

### 3. Data management

Given that each newborn sample is unique and needs to be analyzed with reliability and in a systematic way, data management is an important component of the newborn screening laboratory infrastructure. Typically, the data management system, or laboratory information system (LIS) is expected to manage sample and patient information, interact with the computers onboard both the sample preparation instruments, the analytical instrumentation, manage the various analytical and interpretation algorithms, as well as the production of reports and their transmission to the appropriate health care professional(s). Ideally, the LIS should be able to compute laboratory production statistics, gather and analyze quality control procedures as well as contribute, at least partly, to the laboratory's Quality System indicators. Data storage is usually regulated within each health jurisdiction with respect to its duration, access by various stakeholders and usage. Laboratory data can also be used to evaluate the NBS laboratory's performance. Typically, monitoring of the rate of positive samples (which should remain stable over time), the ratios of various analytical signals within samples, the false positive rate, signal-to-noise ratios can be very useful for laboratory professionals and managers to detect either failures in a laboratory process, or sometimes actual deterioration of the quality of a specific production step or equipment that merit some attention to prevent more serious problems. In the context of laboratories that process all newborn samples from a specific geographic region, particular attention must be placed on preventing a production shutdown which would be very detrimental in the context of diseases that could remain undetected for a longer period of time than acceptable.

### 4. Revised criteria for population screening

The low reagent cost of MS/MS and its capacity to detect several tens of metabolites in a single run have challenged the paradigm of population screening criteria. As discussed above, different countries have introduced MS/MS technology and expanded the number of disorders in their panel at very different rates after applying different policies and approaches. Two such extremes are the UK, which screen only for a few disorders and the USA where most states now screen for 30 disorders and more. Indeed, if the UK health authorities approved MS/MS in principle, apart from PKU and MCADD, no further expansion is envisioned before more data are produced on evidence of benefits of NBS for

other disorders (Pandor et al., 2004). At the other end of the spectrum, most states in the USA now comply to the proposed American College of Medical Genetics (ACMG) core panel of 29 disorders to be screened as primary targets, and an additional 25 disorders (so called secondary targets) that should be identified and reported through full MS/MS profiling (Watson et al., 2006). Many countries, such as Germany, Switzerland and Australia, but also some states in the USA and provinces in Canada, have performed their own evaluation process resulting in expansion to a number of disorders in between both extremes. Clearly, the original screening principles established by Wilson and Junger in 1968 (Wilson & Junger, 1968) - discussed in section 1.2 - have not been applied strictly to NBS. This may be a consequence of the qualitative terms of the Wilson and Junger principles, which make them difficult to use as decision tools. However, the difficulty also arises from the nature of NBS for metabolic diseases which are rare disorders with unclear natural history and benefits of preventive measures/treatment due to desperately lacking data. Consequently, as more disorders with very low prevalence and heterogeneous clinical characteristics are added to the NBS panel, it becomes difficult to establish the balance between potential benefits and harms, as well as to evaluate cost-effectiveness. Interestingly, a recent report showed that parents of children affected with very rare disorders are much in favor of NBS, even for untreatable disorders, mainly because NBS may eliminate a painful diagnostic odyssey (Plass et al., 2010). On the other hand, the recent report from the US Chair on Bioethics unequivocally rejected the technological imperative as a argument for NBS (President's Council on Bioethics, 2008). It is beyond the scope of this chapter to detail the ethical stakes of NBS expansion, but as pointed out by Botkin et al (2006), when neither benefits nor harms are well characterized, a more cautious approach may be warranted.

There is no consensus on whether Wilson and Junger screening principles might or might not be applicable for NBS and the ACMG panel shows a number of flaws (Moyer et al., 2008). Randomized controlled trials may be warranted in NBS, and there is a need to develop a rigorous process to assess available evidence and develop decision tools. Recently, a proposal for reviewed Wilson and Junger screening principles was published in the WHO-bulletin (Andermann et al., 2008). In addition the UK National Screening Committee (NSC) further expanded the 10 Wilson and Junger screening principles into 19 more specific criteria and 87 items of information under 35 general headings (UK National Screening Committee, 2011). These attempts at better defining screening criteria and their evaluation may help public health authorities to better determine which disorders should be implemented into universal public health NBS programs as innovations and new knowledge is reported. Also, cost-effectiveness studies, when available, will inform decision makers about screening options and the impact of various parameters within specific health jurisdictions and population contexts (Venditti et al., 2003, Pandor et al., 2004).

## 5. Perspectives for tandem mass spectrometry in population screening

Tandem mass spectrometry has become a standard method in routine clinical laboratories. The field of newborn screening for inherited metabolic diseases has shown to be a natural fit for this technology that is both sensitive, precise and allows multiplex analyses of several tens of analytes from very small samples. Since year 2000, tens of thousands infants and their families have already benefited from the early identification and treatment of their inherited metabolic disease that has been allowed by this technology. Industry has also rapidly adapted and now proposes turnkey solutions for large panels of relevant (and

perhaps less relevant) NBS metabolites (Lehotay et al., 2011). New methods are proposed on a regular basis and, in the near future, it would not be surprising to witness the availability of MS/MS approaches for the more complex molecules such as the peptides measured in congenital hypothyroidism and hemoglobinopathies. Progresses at automation of many steps of MS/MS in the clinical laboratory (Vogeser & Kirchhoff, 2011), as well as miniaturization of instruments (e.g. by the use of nanosprays and microfluidics) also open new avenues for further improvement of the efficacy of these techniques.

The existence of unresolved questions is highlighted by the great variety in the size of testing panels offered in different jurisdictions, as well as significant discrepancies between existing guidelines for NBS programs with respect to the diseases to be screened. Future applications of this powerful technology will likely include the detection and measurement of peptides and complex molecules with potential applications in NBS. Although other analytical methods will continue to be needed either to screen for specific molecules, or for confirmatory studies of samples positive on screening, tandem mass spectrometry in the field of newborn screening is already a pillar technology and is promised to a great future.

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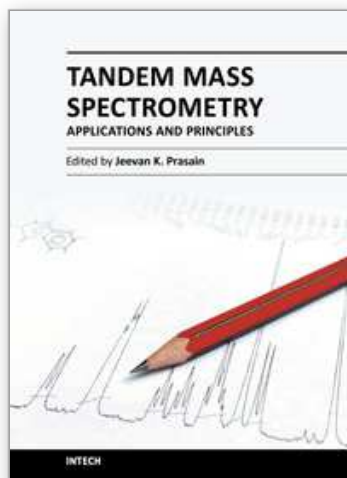
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