Session 8 – Candidate Conditions

Wednesday, Oct. 29 – 1:30pm-3:00pm

Moderators – Joan Scott, MS, CGC, Health Resources Services Administration and Scott Shone, PhD, New Jersey Division of Public Health & Environmental Laboratories

New York's Experience: Summary of the First Four Months of ALD Screening

Abstract

Objective: To assess outcomes for the first months of newborn screening for x-linked adrenoleukodystrophy (ALD) in New York State.

Methodology: First tier screening is performed using MS/MS for C26:0 lysophosphatidylcholine (C26:0 LPC), the primary biomarker used for newborn screening of ALD and other peroxisomal disorders. Second tier screening is accomplished using selective HPLC and MS/MS for C26:0 LPC in infants with a result of ≥ 0.40 µmole/L on the first tier test. Third tier sequence analysis of the coding and promoter regions of the entire ABCD1 gene is done for all infants with C26:0 LPC values ≥ 0.4 µmole/L on the second tier assay. Repeat samples are requested for infants with second tier C26:0 LPC concentrations ≥ 0.24 and < 0.4 µmol/L. Follow-up at 1 of 9 Metabolic Specialty Care Centers is recommended for infants with a positive screen.

Results: During the first 4 months, 75,884 infants were screened for ALD and eight (n=0.011%) were referred for a diagnostic evaluation (6 males, 2 females). Three boys have been confirmed with ALD. Diagnostic evaluation is pending for two additional boys with a positive screen (elevated C26:0 LPC and an ABCD1 mutation). One carrier female was identified with a mutation in ABCD1. One male and one female were referred because of a high C26:0 LPC level; neither baby has an ABCD1 gene mutation detected.

Conclusions: The NYS Newborn Screening Program has a low referral rate, and thus far has successfully detected three infants with ALD in the first four months of screening.

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Summary

Background: X-linked adrenoleukodystrophy is a metabolic disorder affecting the adrenal glands, neurological system and testes (Loes DJ, et al 2003) occurring in 1 in 17,000 births (Dubey, 2005; Raymond, 2007; Schaller, 2007) and 1 in 20,000 males (Mahmood, 2007; McKinney, 2013). There are two main phenotypes: childhood cerebral ALD and the adult onset adrenomyeloneuropathy (Kemp, 2012), which can occur within the same family (Berger, 1994). There is not a correlation between
Adrenoleukodystrophy has been under consideration as a disorder amenable to newborn screening for the last several years. The morbidity and mortality associated with undiagnosed adrenal disease and the emergence of hematopoietic cell therapy (HCT) as a standard therapy for cerebral disease are reasons to consider screening for ALD. (Raymond et al., 2007, Peters, 2004) Newborn screening allows for diagnosis of ALD prior to the onset of adrenal and neurological symptoms. There is usually biochemical evidence of adrenal insufficiency at the time of diagnosis of ALD, when siblings of a proband are tested. (Dubey et al., 2005) Cox et al demonstrated the “window of opportunity” for treatment of cerebral disease in ALD (Cox, 2014). In their study, thirty out of thirty-two boys with ALD and normal MRIs had normal cognitive function. Although there is a “window of opportunity,” 50% of clinically diagnosed patients with cerebral disease are already at an advanced stage of the disease (Schaller, 2007).

Moser et al in 1981, discovered that very long-chain fatty acids (VLCFA) are elevated in plasma from males with adrenoleukodystrophy (Moser, 1981; Kemp, 2012; Schaller, 2007) and are elevated in up to 80-85% of heterozygotes. (Kemp, 2012; Raymond, 2007; Schaller et al., 2007) In 2006, Hubbard et al described a combined LC-MS/MS Method for analysis of VLCFA in dried blood spots (Hubbard, 2006). In 2011, Sandlers et al developed a one-step extraction procedure that simultaneously extracts acylcarnitines and the lysophosphatidyl cholines (C26:0-LPC) from DBS with a minute MS/MS run time (Sandlers, 2012). Methanol extracts can be analyzed with flow-injection MS/MS, however, there are isobaric compounds that interfere in the quantitation of C26:0 LPC, hence, an HPLC-MS/MS assay must be performed to accurately quantify the C26:0 LPC level in dried blood spots. Matern (Mayo, Rochester, MN, personal communication) combined methanol extracts for ALD screening with extracts used in lysosomal storage disease screening; this method was shared with New York. Together, all these advances in screening methods removed a major barrier to the implementation of newborn screening for ALD (ALD NBS).

On April 29, 2012, New York resident Aidan Seeger died from ALD. His mother, Elisa Seeger is an advocate for ALD NBS. The combination of an improved DBS assay and advocacy, allowed for consideration of ALD NBS by NYS legislators and the NYS NBS Program. On March 31, 2013, Aidan’s Law was signed mandating the New York State Newborn Screening Program to begin screening for the disorder. On December 30, 2013 newborn screening for adrenoleukodystrophy commenced in NYS.

ALD NBS in NYS is accomplished using a three-tier algorithm. The first tier is MS/MS of C26:0 LPC, this testing is performed in reflux mode with Krabbe disease screening (extracts from Krabbe assay are combined with ALD extracts) and the second tier is the C26:0 LPC using HPLC MS/MS (Hubbard, 2006 and 2009). The third tier involves sequencing of the ABCD1 gene, which was identified in by Mosser in 1993. (Kemp, 2012; Mosser, 1993) Each of the 10 exons are sequenced (Kemp, 2012).

As of September 9, 2014, 165,142 infants were screened for ALD, of these 3,609 samples had elevated C26:0 LPC by the first tier MS/MS assay and went for second tier HPLC-MS/MS testing. Of these, 14 infants had elevated C26:0 LPC concentrations and third tier DNA testing was performed. DNA testing is used only to determine if there are mutations in the ABCD1 gene, infants are referred for follow-up diagnostic testing regardless of the results obtained in DNA testing. Seven male infants and three female infants were determined to have a mutation in the ABCD1 gene. The remaining 4 infants are under evaluation but at least two were determined to have Zellweger spectrum disorder.
There are challenges to screening for ALD and screening simultaneously with Krabbe disease screening further complicates the screening; challenges include the limited solubility of the internal standard (d4-C26:0 LPC) and ionization suppression effect from the ALD extract on target lysosomal storage disorder markers. The challenges have been primarily overcome and improvements to the combined assay are continually being made. Thus far, our screen positive rate is 1 in 11,796 infants screened, and the predicted incidence of ALD in males is 1 in 11,796 assuming that all males with elevated marker and a mutation in the ABCD1 gene become symptomatic. The overall incidence rate of ALD (males and females) is 1 in 23,591. The incidence rates are very preliminary, and a major drawback to screening infants for ALD is that the positive predictive value of the test will not be known for many years, perhaps decades, due to the expected delay in symptoms associated with ALD. Long-term follow-up of screen positive cases of ALD must be completed to completely understand the effectiveness of this screening.

References:
Missouri's Experience with Full Population Pilot Screening for Pompe, Gaucher, Fabry and Hurler Disorders using Digital Microfluidics Methodology
P. Hopkins, Missouri State Public Health Laboratory, Jefferson City, MO

Abstract

In January, 2013, Missouri (MO) began a full population pilot/implementation phase to screen for Pompe, Fabry, Gaucher and Hurler disorders using the digital microfluidics (DMF) multiplex enzymatic assay technology provided by Advanced Liquid Logic, Inc., an Illumina Company (ALL).

For this LSD pilot phase, the MO State Public Health Laboratory set up 2 work stations, each with 4 DMF instruments. Full population pilot screening began on January 11th, 2013, where all routine newborn screening (NBS) samples received were assayed for the 4 LSDs. On the second day of the testing, a positive Pompe screen was referred to our contracted referral center and was subsequently confirmed with infantile Pompe through molecular and other diagnostic testing. The infant was promptly placed on enzyme replacement therapy and is doing well one year later.

To date, over 118,000 newborn samples have been screened in MO with 187 being identified as screen positive and referred to specialists for evaluation and confirmation. 45 of these infants were confirmed as having LSD genotypes; 8 with Pompe (3 infantile, 5 late onset), 26 with Fabry, 1 with Gaucher and 1 with Hurler. 9 other positive screens were confirmed as having LSD genotypes of unknown significance or onset through molecular and enzyme testing. These 9 infants are currently asymptomatic but are continually being followed. 23 others were confirmed to have pseudodeficiency conditions and are not affected, and 13 others were found to be carriers. 82 were false positive. Currently, 24 referred screen positive cases are pending final confirmation.

The DMF method has performed very well for MO’s screening of 90,000 samples per year with a positive predictive value averaging 24% with no known missed cases to date. Good communication with our LSD taskforce and genetic referral centers has promoted ongoing improvements in our cutoffs and decision schemes. This presentation will provide up to date statistics at the NBSGTS along with relevant data and lessons we have learned.

Screening for Krabbe and Niemann-Pick via DMF will be added in the near future as appropriate screening tests are being developed by ALL. MO continues to contract with the New York NBS program to screen MO’s samples for Krabbe since August 2012. An update regarding those statistics will be provided in this presentation also.

Presenter: Patrick Hopkins, BS, Missouri State Public Health Laboratory, Newborn Screening Unit, Jefferson City, MO, Phone: 573.751.1418, Email: Patrick.Hopkins@health.mo.gov
Summary

In January, 2013, Missouri (MO) began a full population pilot/implementation phase to screen for Pompe, Fabry, Gaucher and Hurler disorders using the digital microfluidics (DMF) multiplex enzymatic assay technology provided by Baebies Inc., formerly Advanced Liquid Logic, Inc. Missouri chose to utilize this methodology due to space, cost and time constraints that were being faced at the time, and was attracted by the ease at which this methodology could be incorporated into the current newborn screening laboratory.

For this LSD pilot phase, the MO State Public Health Laboratory required 2 work stations, each with 4 DMF instruments. After a full IRB review and a three month pre-pilot and validation phase, full population pilot screening began on January 11th, 2013 where all routine newborn screening (NBS) samples received were assayed for the 4 LSDs.

Two scientists are currently able to fully conduct the LSD testing, interpretation and reporting, however 4 others are cross-trained in this testing area for backup when necessary. NBS samples are punched using single DBS punch machines at approximately 15 minutes per 96-well plate. Only one punch is needed for the 4-plex assay. Extraction takes 30 minutes at room temperature, while cartridges are prepared by filling with the oil matrix. There are 48 sample wells per cartridge and one cartridge is run at a time on each instrument. Each cartridge has 10 controls (2 blanks, 4 calibrators, 2 low controls and 2 medium controls) and 38 patient samples. Sample loading takes approximately 5 minutes. Machine run time is approximately 2.5 hours for the 4-plex assay. Total time from sample punch to enzyme activity results is approximately 4 hours.
High risk positive screens would be referred to one of Missouri’s four contracted metabolic referral centers. The designated referral center would contact the primary care physician and a plan would then be developed to conduct confirmatory testing and treatment/management for the baby based on developed guidelines from the MO NBS program.

On the second day of the testing, a positive Pompe screen was referred to our contracted referral center and was subsequently confirmed with infantile Pompe through molecular and other diagnostic testing. The infant was promptly placed on enzyme replacement therapy and is doing well with meeting normal developmental milestones.

Cutoffs for flagging abnormal enzyme activities and referrals of positive screens were set conservatively at the beginning of the pilot. Monthly LSD task force conference calls consisting of the State NBS Laboratory, the follow-up program and the contracted referral centers were conducted to provide updates and valuable feedback on the progress of the LSD screening. Certain cutoff levels were slightly adjusted on six occasions throughout the first year of testing.

To date, over 150,000 newborn samples (approximately 130,000 births) have been screened in MO with 240 being identified as screen positive and referred to specialists for evaluation and confirmation. 47 of these infants were confirmed as having LSD genotypes; 12 with Pompe (5 infantile, 7 late onset), 33 with Fabry, 1 with Gaucher and 1 with Hurler. 11 other positive screens were confirmed as having LSD genotypes of unknown significance or onset through molecular and enzyme testing. These 11 infants are currently asymptomatic but are continually being followed. 37 others were confirmed to have pseudodeficiency conditions and are not affected, and 13 others were found to be carriers. 91 were false positives, however some of the false positives could in actuality have been carriers, as several of
these infants displayed low-normal ranges in confirmatory diagnostic enzyme testing and therefore were closed as normal without further DNA testing to identify carrier status. Currently, 33 referred screen positive cases are pending final confirmation.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Screened Positive</th>
<th>Confirmed Disorder</th>
<th>Condition Currently of Unknown Significance or Onset</th>
<th>Pseudo-deficiency</th>
<th>Carrier</th>
<th>False Positive*</th>
<th>Lost to Follow-up</th>
<th>Pending</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pompe</td>
<td>65</td>
<td>12 (5 infantile, 7 late)</td>
<td>3</td>
<td>11</td>
<td>9</td>
<td>17</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Gaucher</td>
<td>19</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>11</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Fabry</td>
<td>94</td>
<td>33</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>38</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Hurler</td>
<td>62</td>
<td>1</td>
<td>0</td>
<td>26</td>
<td>3</td>
<td>25</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Aggregate</td>
<td>240</td>
<td>47</td>
<td>11</td>
<td>37</td>
<td>13</td>
<td>91</td>
<td>8</td>
<td>33</td>
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</tbody>
</table>

Other important laboratory findings obtained from this LSD full population pilot phase have been that LSD enzyme activities drop slightly during the first 2 weeks of age and then stabilize after 14 days-of-age. The use of age-related cutoffs for older babies has been necessary. Our data has shown that premature babies can have altered LSD enzyme levels. Repeat screens may be more reliable on this subpopulation. We have seen that multiplexing the four LSD assays greatly helps assess the quality of the NBS sample and the risk for referral as all four enzyme levels can be observed together. Some seasonal variation is observed with enzyme activities, similar to the galactosemia (GALT) assay in that more carriers, pseudo-deficiencies and false positives can be detected during higher heat and humidity months. This seasonal phenomenon may be isolated or sporadic and not necessarily observed across the full spectrum of samples received during the warmer season.

In conclusion, the digital microfluidics multiplex method has performed very well for MO’s screening of 90,000 samples per year with a positive predictive value averaging 29% with no known missed cases to date. The method was easily incorporated into the Missouri NBS laboratory and was easy to cross-train many staff to conduct. Good communication with our LSD taskforce and genetic referral centers has promoted ongoing improvements in our cutoffs and decision schemes.

MO continues to contract with the New York NBS program to screen MO’s samples for Krabbe Disease since August 2012. Baebies Inc. are working on developing fluorimetric assays for Krabbe and Niemann-Pick for Missouri to incorporate into their LSD testing program.
Enzymatic Assays for Newborn Screening of Mucopolysaccharidoses with Improved Performance
A.B. Kumar, S. Massi, N.K. Chennamaneni, M. Barcenas, F. Turecek, C.R. Scott and M.H. Gelb, University of Washington, Seattle, WA

Abstract

We have previously published tandem mass spectrometry (MS/MS) assays for lysosomal enzymes that are appropriate for newborn screening of mucopolysaccharidoses (MPS-I, MPS-II, MPS-IIIA-D, MPS-IVA- and MPS-VI) using dried blood spots on newborn screening cards. More recently, we have improved these assays by developing a new set of reagents that lead to greatly improved sensitivity. This is especially important for the enzyme relevant to MPS-IVA, since activity of this enzyme with traditional substrates is extremely low. The assay of iduronidase for newborn screening of MPS-I is done as a part of a new 6-plex assay being commercialized by Perkin Elmer. In this poster, we report the multiplex analysis of MPS-II, IIIA, IIIB, IVA, and VI using a single 3 mm punch of a dried blood in a single assay cocktail. Prior to mass spectrometry, samples are processed through a very simple liquid-liquid extraction protocol followed by solvent removal and direct infusion into the tandem mass spectrometry (no liquid chromatography required). The new assays for the enzymes relevant to mucopolysaccharidoses out-perform all other reported assays by at least an order of magnitude in terms of assay sensitivity and discrimination between affected and non-affected individuals. We have also developed a new fluorimetric assay of the enzyme relevant to MPS-IVA that out-performs the previously reported fluorimetric assay in terms of sensitivity. We have also developed a fluorimetric assay of the enzyme relevant to MPS-VI. No fluorimetric assay of this enzyme has been reported before.

Pilot newborn screening studies are in progress with these new assays in order to explore the feasibility of newborn screening for the set of mucopolysaccharidoses for which treatment is available or in development.

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Summary

Substrates for MS/MS assays of the enzymes relevant to MPSs are shown in Figure 1. The enzyme acts on the substrate to remove either the sugar (MPS-I) or the sulfate attached to the sugar (MPS-II, -IIIA, -IVA, -VI). The source of the enzyme is a dried blood spot on newborn screening cards. The reagents are designed with multiple features: 1) Ease of synthesis; 2) Close resemblance to the natural enzyme substrate; 3) Functionality in the enzymatic product that allows ease of protonation for formation of a mono-cation for analysis by electrospray ionization MS/MS; 4) High yield extraction of the enzymatic production into ethyl acetate for simple pre-MS/MS sample processing; 5) Ease of incorporation of heavy isotopes for preparation of internal standards that are chemically identical to the enzymatic products.
**MPS-II.** Mucopolysaccharidosis type-II (MPS-II) is caused by the deficiency of the enzyme iduronate 2-sulfatase. As shown in Scheme 1 the disease is identified by quantifying the MPS-II product formed using MS/MS.

![Scheme 1. Iduronate 2-sulfatase reaction for MS/MS analysis of MPS-II](image)

In a modified assay, we add recombinant alpha-iduronidase, which acts on MPS-II product but not MPS-II substrate, to give the aglycone (product without the sugar, not shown). Detection of the aglycone rather than MPS-II product provides a dramatic improvement of detection sensitivity by MS/MS.

**MPS-IVA.** Deficiency of the enzyme galactose 6-sulfate sulfatase results in MPS-IVA (the same enzyme also acts on N-acetyl-galactosamine-6-sulfate). We have developed two forms of the assay for measuring the enzyme activity. In the first method, the MPS-IVA product (Scheme 2) formed by the action of galactose 6-sulfate sulfatase on MPS-IVA substrate is directly measured by MS/MS. We make use of a novel substrate that contains an N-acetyl-galactosamine-6-sulfate in place of galactose-6-sulfate as we found that the former is greatly preferred by the MPS-IVA enzyme. This leads to a dramatic increase in product formation compared to all previously reported substrates. Hexosaminidase A present in the dried blood spot can act to degrade both MPS-IVA substrate and MPS-IVA product, thus we include a specific inhibitor to block this reaction. In a second method we use a novel bacterial enzyme, NGA, to selectively cleave N-acetyl-galactosamine from MPS-IVA product; NGA does not act on MPS-IVA substrate and is not inhibited by the hexosaminidase A inhibitor. The action of NGA forms aglycone 1 (Scheme 2), which extracts into ethyl acetate for detection by MS/MS.
Scheme 2. Methods 1 and 2 for the MPS-IVA assay

The use of NGA allows us to carry out a novel fluorimetric assay of the MPS-IVA enzyme by using the glycoside of N-acetylgalactosamine-6-sulfate with 4-methylumbelliferone. This assay greatly outperforms the previously reported fluorimetric assay based on the glycoside of galactose-6-sulfate and 4-methylumbelliferone since it is based on a greatly preferred substrate.

MPS-VI. Scheme 3 shows two methods for assaying the N-acetyl-galactosamine 4-sulfatase for analysis of MPS-VI. The methods are analogous to our new MPS-IVA assays (Scheme 2). MPS-VI product is either detected directly, or we use NGA to convert MPS-VI product to aglycone 2, leading to improved product detection sensitivity by MS/MS.

Scheme 3. Methods 1 and 2 for the MPS-VI assay

The final datasets for these MS/MS assays is still being finalized at the time of this summary, but will be presented at the 2014 APHL Newborn Screening Meeting in Anaheim, CA. Briefly, we obtain 200,000-300,000 product ion counts in the MPS-II assay compared to 2,000-3,000 counts in the no-blood control. For MPS-IVA we obtain ~50,000 ion counts of product signal compared to ~3,000 for the no blood control. The values for MPS-VI are ~80,000 and ~2,000 with and without blood, respectively. All assays Proceeding of the 2014 APHL Newborn Screening and Genetic Testing Symposium, Anaheim, CA, October 27-30, 2014
are done with an overnight incubation, but can also be done with a 2-3 hr incubation if desired. We will show that these MS/MS assays greatly out perform all previously reported assays of these enzymes including fluorimetric assays using standard and digital microfluidics methods.

A Pilot study of MPS-I is currently underway in the newborn screening lab in WA state to reach ~100,000 DBS over 1.5-2 years. Pilot studies for MPS-VI are in the planning stages and will start soon WA state and Taiwan). PerkinElmer Genetics is gearing up to provide all of these assays to the medical community upon demand. Reagents for other testing labs to establish these assays will be made available in the next few months. We are also developing an MS/MS assay for MPS-VII.

Funded by grants from the NIH, Biomarin, and Shire.

A 3 Year Pilot study for Guanidinoacetate Methyltransferase (GAMT) Deficiency in British Columbia
G. Sinclair, C. Van Karnebeek, T. Nelson, H. Vallance, S. Stockler, British Columbia Children's Hospital/University of British Columbia, Vancouver, BC, Canada

Abstract

Introduction: Guanidinoacetate methyltransferase (GAMT) deficiency is a rare disorder of creatine biosynthesis presenting with epilepsy and developmental delay in infancy with progressive neurodegeneration if untreated due to cerebral creatine deficiency and guanidinoacetate (GAA) toxicity. Several case reports have shown excellent outcomes for infants identified and treated from birth due to a family history. In October 2012 the BC Newborn Screening Program initiated a 3 year pilot screening study for GAMT deficiency to evaluate the disease incidence and the performance of a novel three-tiered screening assay.

Methods: All bloodspot samples submitted for routine newborn screening in BC have been included in the pilot study as de-identified but linkable specimens. GAA is initially measured by flow injection mass spectrometry using a modification to our routine acylcarnitine/amino acid first-tier assay. All samples with values above the 99.8th%ile are reflexed to a more specific LC-MS/MS GAA assay integrated into our MSUD second-tier analysis. GAMT gene sequencing is then completed on the original newborn bloodspot for all specimens with elevated GAA on the second-tier test. Any samples with one or two GAMT mutations are to be re-identified so that appropriate follow-up and treatment can be initiated.

Results: From October 2012 to date, over 71,000 specimens have been tested. 120 samples have been above the first-tier screening cut-off and one sample had slightly elevated results on the second-tier GAA analysis. Molecular testing identified only the normal sequence in that sample, it was deemed a false positive and no follow-up was initiated. Based on historical rates of clinical ascertainment in BC, we anticipate a single true positive GAMT case over the study period.

Presenter: Graham Sinclair, PhD, FCCMG, British Columbia Children's Hospital/University of British Columbia, Pathology and Laboratory Medicine, Vancouver, BC, Canada, Phone: 604.875.2345 x7450, Email: gsinclair@cw.bc.ca
**Summary**

**Introduction:** Guanidinoacetate methyltransferase (GAMT) deficiency is one of three known inherited cerebral creatine deficiency disorders. Creatine is synthesized from arginine and glycine through an intermediate, guanidinoacetate (GAA), by the sequential activities of arginine:glycine amidotransferase (AGAT) and GAMT enzymes. Deficiencies in either of these enzyme or the X-linked creatine transporter (SLC6A8) lead to a deficiency in cerebral creatine levels. This deficiency results in early global developmental delay with progressive neurodegeneration and epilepsy if untreated. In GAMT deficiency, GAA toxicity is also implicated in the pathophysiology of disease.

Over 80 cases of GAMT deficiency have been reported in the literature since the discovery of this disorder in 1994, but the true incidence of the disease remains unclear. Selective screening for creatine deficiency disorders in a cohort of French patients with unexplained neurological dysfunction identified GAMT deficiency in ~1/1000 individuals; however this was a highly selected group and does not represent a population incidence. A founder effect in Portugal has lead to an incidence of 1/60,000 in that country but no other incidence figures are available, suggesting that this disorder is either rare or underdiagnosed.

Given the underlying biochemistry of GAMT deficiency, a number of rational treatment approaches have been employed. A recent review of 48 treated patients from around the world has shown improved outcomes with combinations of creatine and ornithine supplementation, dietary arginine restriction, and in some cases, the addition of sodium benzoate as a glycine scavenger. Such approaches have been shown to normalize CSF creatine levels and reduce toxic accumulations of GAA in both CSF and plasma. Despite the biochemical improvements, however, clinical improvements have been variable, correlating strongly with age at initiation of therapy. Older patients have shown reductions in seizure activity and a halting of disease progression, but few improvements in existing intellectual disabilities. In contrast, those infants treated from birth due to a previous family history have shown normal or near-normal intellectual development. Although there are only a handful of such cases worldwide, these positive outcomes have lead authors to argue strongly for newborn screening for GAMT deficiency.

**Newborn Screening:** Newborn screening for GAMT deficiency has been trialed in a number of jurisdictions with variable outcomes. An initial trial in Austria suffered from a high false positive rate and was terminated (Stockler S, personal comm). Similarly high false positive rates also affected a trial in Portugal, although a successful long-term screening program in Australia has recently been reported. More recently, a variety of multi-tiered approaches to screening have been proposed and trialed in BC, Utah, Italy, and Texas. Adding a second-tier LC-MS/MS assay for GAA quantitation from bloodspots removes the interference seen in standard flow injection assays for some newborns, greatly improving test performance. Despite these analytical improvements, implementation of routine GAMT screening remains limited and to the best of our knowledge, no affected infants have been identified through newborn screening. In October 2012, the British Columbia Newborn Screening Program initiated a 3-year pilot screening study for GAMT deficiency to evaluate the disease incidence and the performance of a novel three-tiered screening assay.

**Methods:** All bloodspot samples submitted for routine newborn screening in BC have been included in the pilot study as de-identified but linkable specimens. This is a non-consented pilot but families are informed of the study through a newborn screening pamphlet and information on our website. Approval for this linkable approach was granted by the UBC C&W Research Ethics Board and the BC Newborn screening Advisory and Research Review committees to allow for therapeutic intervention.
should an affected infant be identified during the pilot. GAA is initially measured by flow injection mass spectrometry using a modification to our routine acylcarnitine/amino acid first-tier assay. All samples with values above the 99.9th percentile are reflexed to a more specific LC-MS/MS GAA assay integrated into our MSUD second-tier analysis. GAMT gene sequencing is then completed on the original newborn bloodspot for all specimens with elevated GAA on the second-tier test. Any samples with one or two GAMT mutations are to be re-identified and referred to a metabolic specialist so that appropriate follow-up and treatment can be initiated.

**Results:** From October 2012 to date, over 88,000 specimens have been tested. The first-tier assay has shown acceptable performance with CV <10% at low and high QC levels. GAA values for the population have been in keeping with published values (GAA = 1.65 ± 0.55). GAA levels have been above the first-tier screening cut-off of 6uM for 122 newborns (0.14%). The second-tier LC-MS/MS assay separates out an interfering substance and has yielded GAA values below 5 uM for all but one specimen (GAA = 6.8 uM). Molecular testing identified only the normal sequence in that sample, it was deemed a negative screen, and no follow-up was initiated as per the study protocol.

**Discussion:** An integrated three-tier approach to GAMT screening has shown acceptable test performance with zero false positive results after 2 years of screening on 88,000 specimens. Both the first and second-tier tests have been integrated into existing laboratory assays minimizing the cost of screening for this apparently rare disorder. GAA elevations seen on the first-tier screening test, due to an isobaric interfering compound (yet to be identified), are successfully corrected by the second-tier LC-MS/MS assay. Of note, the abundance of this interference appears to be age-dependent as noted by an over-representation of late collected samples (>7 days) in the first-tier positive group. Most of these represent routine requested recollections for low birth weight or transfusion. This is in keeping with the findings of Pitt et al (2014) who speculate that this interference results from a therapeutic intervention administered to premature infants.

Although GAMT deficiency is a rare disorder without a clear population incidence, two-tier GAA biochemical screening can be integrated into existing assays with minimal cost or added labour. The inclusion of the third-tier dried bloodspot GAMT gene sequencing step has been able to reduce the false positive rate to zero, further minimizing the potential harms of screening. Given the promising outcomes from early therapeutic intervention, GAMT deficiency remains an excellent candidate for routine newborn screening.

**Funding:** Rare Diseases Foundation, Vancouver Canada and mCentred Woman’s Organization, Vancouver Canada.

**References:**