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A three-tier algorithm for guanidinoacetate methyltransferase (GAMT) deficiency newborn screening☆

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ABSTRACT

Background: Guanidinoacetate methyltransferase (GAMT) deficiency is a rare disorder of creatine biosynthesis presenting with epilepsy and developmental delay in infancy. Excellent developmental outcomes have been reported for infants treated from birth due to a family history. The BC Newborn Screening Program initiated a 3 year pilot screening study for GAMT deficiency to evaluate the performance of a novel three-tiered screening approach.
Methods: Over 36 months all bloodspots submitted for routine newborn screening were included in the pilot study (de-identified). Initial GAA measurement was integrated into the standard acylcarnitine/amino acid first-tier assay. All samples with elevated GAA were subjected to second-tier GAA analysis by LC-MS/MS integrated into an existing branched-chain amino acid (MSUD) method. GAMT gene sequencing was completed on the original bloodspot for all specimens with elevated GAA on the second-tier test. The protocol allowed for re-identification for treatment of any specimen with one or two likely pathogenic GAMT mutations.
Results: Over the study period 135,372 specimens were tested with 259 (0.19%) over the first-tier GAA cut-off. The second-tier assay removed an interference falsely elevating GAA levels, and only 3 samples required genotyping. No mutations were identified in any samples, all were deemed negative screens and no follow-up was initiated.
Conclusions: A three-tier algorithm for GAMT newborn screening showed excellent test performance with zero false positives. No cases were detected, supporting a low incidence for this disorder. Given the low incremental costs and evidence of positive outcomes with early intervention, GAMT deficiency remains an excellent candidate for newborn screening.

1. Introduction

Guanidinoacetate methyltransferase (GAMT) deficiency is an autosomal recessive condition due to bi-allelic mutations in *GAMT* (MIM 602140), and one of three known inherited cerebral creatine deficiency disorders [1]. Creatine is synthesized from arginine and glycine through an intermediate, guanidinoacetate (GAA), by the sequential activities of arginine:glycine amidinotransferase (AGAT) and GAMT enzymes. Deficiencies in either of these enzymes 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 [1].

Over 80 cases of GAMT deficiency have been reported in the literature since the discovery of this disorder in 1994, and although some small scale carrier detection studies have been completed, the true incidence of the disease remains unclear [2,3,4]. Selective screening for creatine deficiency disorders in a cohort of French patients with unexplained neurological dysfunction identified GAMT deficiency in

Abbreviations: GAMT, guanidinoacetate methyltransferase; BC, British Columbia; GAA, guanidinoacetate; LC-MS/MS, liquid chromatography tandem mass spectrometry; MSUD, maple syrup urine disease; AGAT, arginine:glycine amidinotransferase; ddH_20, distilled deionized water; ACN, acetonitrile; ACMG, American college of medical genetics; *Human gene*: *GAMT* (HGNC:416), guanidinoacetate *N*-methyltransferase; *Human gene*: *SLC6A8* (*HGNC:11055*), solute carrier family 6 (neurotransmitter transporter), member 8.

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~1/1000 individuals; however this was a highly selected group and does not represent a population incidence [5]. Although a founder effect in Portugal has led to an incidence of 1/60,000 in that country, several pilot molecular screening studies in other populations have calculated highly variable, but significantly lower carrier frequencies suggesting a low overall disease incidence [3,4,6].

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 [7]. 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 this treatable intellectual disability, GAMT deficiency [8,9].

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 (Stoeckler S, personal communication). Similarly high false positive rates also affected a trial in Portugal, although a successful long-term screening program in Australia has recently been reported [10]. More recently, a variety of multi-tiered approaches to screening have been proposed and trialed in British Columbia (BC), Utah, Italy, Netherlands, and Texas [3,11,12]. 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. Utah went live with state-wide GAMT screening using such a two-tiered approach in July 2015 [13]. 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. Given the apparent low incidence, very high test-specificity would be required to maintain a low false positive rate and high positive predictive value for GAMT deficiency screening. In October 2012, the BC Newborn Screening Program initiated a 3-year pilot screening study for GAMT deficiency to evaluate the performance of a novel three-tiered screening assay for this apparently rare but highly treatable disorder.

2. Materials and methods

2.1. Ethics

All bloodspot samples submitted for routine newborn screening in BC were included in the pilot study as de-identified but linkable specimens. This was a non-consented pilot but families were informed of the study through a newborn screening pamphlet provided at the time of sample collection and information on the program website. The samples were de-identified for testing but were linkable to patient identifiers if the screen result was deemed positive after the third-tier of the screening algorithm. Approval for this 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.

2.2. Screening algorithm

All submitted bloodspot cards were tested for GAA on the first tier assay, integrated into our existing flow injection tandem mass spectrometry (FIA-MS/MS) method for amino acids and acylcarnitines. All samples above the screening cutoff for the first-tier assay (set initially

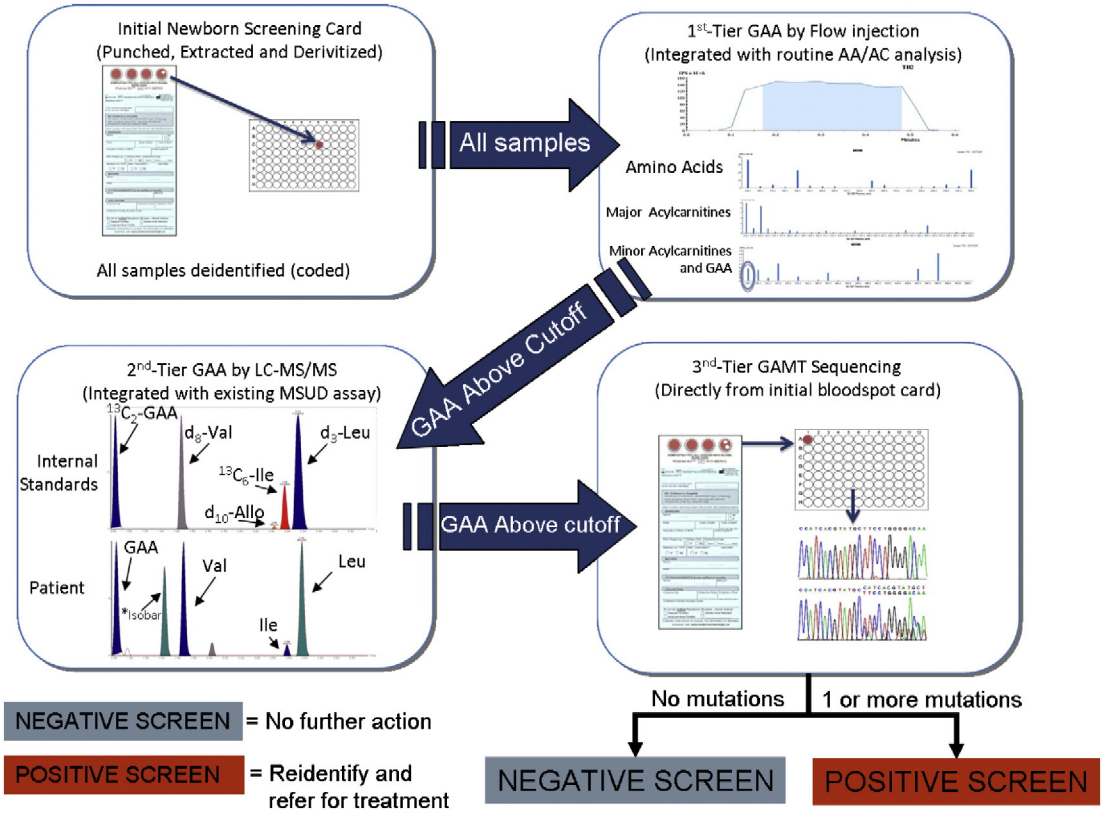

Fig. 1. Three-tiered GAMT screening algorithm.

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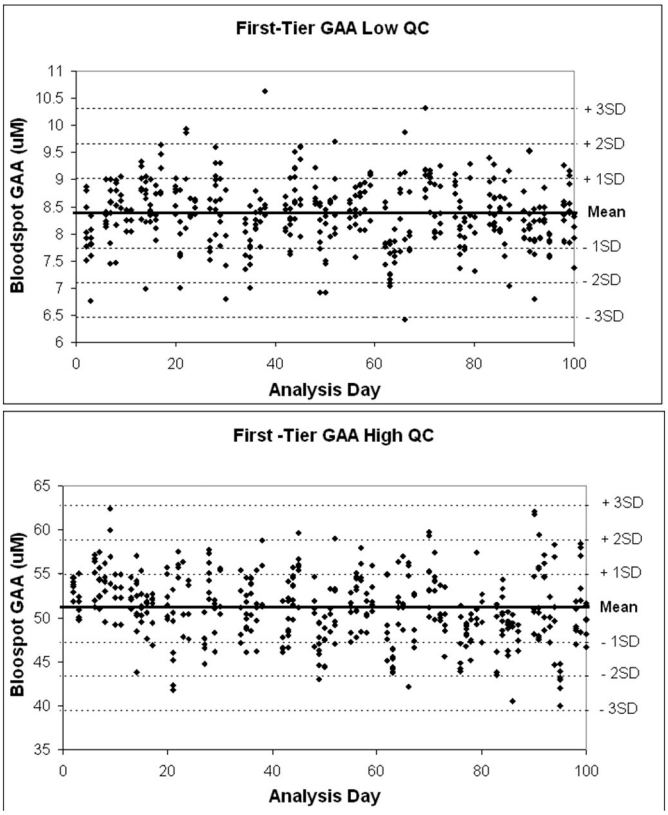

Fig. 2. First-tier GAA Quality Control over a 100 day period.

at the population 99.9th%ile and later lowered to the 99.5th%ile) were repunched in duplicate (two 3 mm punches) and reanalysed to confirm the high first-tier result. All those with a consistently high value continued to the second-tier assay which added column chromatography to separate GAA from an interfering peak (see Fig. 1). This second-tier assay was run using residual material from the first-tier assay, eliminating the need to repunch bloodspot cards and was also integrated into an existing second-tier assay for maple syrup urine disease (MSUD).

The lowest published value for bloodspot GAA in a known GAMT deficiency case was 9 μM at the outset of this pilot [12]. A conservative cut-off GAA value of 6.0 μM (99.9th%ile) was initially utilized for the first-tier assay to minimize the false positive rate but was lowered to 3.5 μM (99.5th%ile) at 18 months given the publishing of a confirmed case of GAMT deficiency with a newborn GAA = 6.5 μM [14]. This cut-off of 3.5 μM was consistent with another published trial utilizing a similar analytical approach [12]. Any sample with consistently elevated GAA following the second-tier assay was punched again for DNA extraction (2 × 3 mm punches). Full sequencing of all exons and flanking intronic sequences of *GAMT* was completed for these cases.

If no sequence variants were identified, this was considered a negative screen. If either one or two (possibly) pathogenic sequence variants were identified in *GAMT*, these would be considered positive screens leading to re-identification of the infant and immediate referral to a metabolic specialist for confirmatory testing and management. Variants identified were classified by a clinically-certified Molecular Geneticist according to ACMG standards and guidelines, with the final interpretation including the bloodspot GAA level as part of the risk assessment [15].

2.3. First tier testing

GAA was initially measured by flow injection mass spectrometry using a modification to our routine acylcarnitine/amino acid first-tier assay, as previously described with minor modifications [11]. Analytes were extracted from 3 mm punches of the dried bloodspots in 200 μl of 80:20 methanol:ddH_20 containing appropriate stable-isotope internal standards (Cambridge Isotopes). Extraction time was 60 min at 60 °C. A set of dried bloodspot external calibrators, prepared for all major acylcarnitine and amino acid species, were analyzed in each batch and individual recoveries corrected by the slope of the calibration curve. Unlabeled acylcarnitines, amino acids, and guanidinoacetate were purchased from Sigma Aldridge or Dr. Herman ten Brink. All minor species were corrected using the slope from a major species of similar mass. Extracted acylcarnitines and amino acids were evaporated to dryness and butylated using butanolic-HCl for 20 min at 60 °C. Samples were again evaporated to dryness and resuspended in 200 μl of mobile phase (80:20 ACN:ddH_20 with 10 mM ammonium formate). 10 μl of this derivitized extract was injected for analysis by flow injection tandem mass spectrometry utilizing a Waters Xevo-TQ tandem mass spectrometer (MS/MS) for MRM data acquisition of the butyl esters (Waters Canada) Butyl-GAA (174.1 > 101.1) was quantitated relative to a butyl ester of 13C2-GAA (176.1 > 103.1) with recovery corrected by a 7 point GAA calibration curve (0–50 μM).

2.4. Second tier testing

The second tier GAA method was integrated into an existing LC-MS/MS method for branched chain amino acid analysis (MSUD screening). Each morning, all calibrators, quality control specimens and patient samples with high branched-chain amino acids or elevated GAA from the overnight first-tier assay were flagged. The assay plates containing the residual extracted and derivitized amino acid, acylcarnitine and GAA mixture for the flagged specimens were evaporated to dryness (15 min 60 °C). The dried extract was then resuspended in 100 ul of a 90:10 ddH2O:ACN solution containing 0.1% formic acid. After 10 min of shaking at 900 rpm, 10 ul of each sample was injected for analysis by LC-MS/MS using a Xevo TQD LC-MS/MS system (Waters Canada). Separation was obtained with a stepped gradient from 85% Buffer A (0.1% formic acid in ddH_20) to 50% Buffer B (0.1% formic acid in ACN) over 5 min on a BEH C18 1.7 μM, 2.1 × 100 mm column (Waters Canada). GAA levels were calculated against the aforementioned 7-point calibration curve.

2.5. Third tier testing

Molecular analysis of *GAMT* was then completed using DNA extracted from the original newborn bloodspot for all specimens with elevated GAA on the second-tier test. DNA was extracted from 2 × 3 mm punches of the original newborn screening bloodspot card, using the QIAamp DNA Investigator kit (Qiagen). All exons plus 10 bp of flanking intronic sequence of *GAMT* (RefSeq NM_000156.5) were amplified by polymerase chain reaction using HotStar Taq (Qiagen). Primer sequences and details of PCR conditions are available upon request. PCR products were Sanger sequenced using the BigDye terminator v3.1 cycle sequencing kit and the 3130xl capillary electrophoresis instrument (Applied Biosystems by Thermo Fisher Scientific). Sequence analysis was carried out using SeqPilot SeqPatient software (JSI Medical Systems). Variant interpretation was outlined as above.

3. Results

3.1. Analytical performance

During 36 months from October 1, 2012 to Sept. 30, 2015, 135,372 newborn bloodspot specimens were tested. The first-tier assay displayed acceptable performance with CV <10% at low (GAA = 8 μM) and high (GAA = 45 μM) QC levels (Fig. 2). The calibration was linear across the measured range with an average slope of 1.2, intercept of 0.1, and $R^2 = 0.999$. GAA values for the population were in keeping

with published values with a mean GAA concentration of 1.54 ± 0.45 μM (Table 1). There were no significant trends with respect to birth weight or age at collection as previously reported by others (data not shown). [12] The second-tier assay showed similar acceptable performance metrics with CV < 10% at both QC levels and a similarly linear calibration.

3.2. Screening results

GAA levels were above the first-tier screening cut-off of 6 μM (first 18 months) or 3.5 μM (final 18 months) for 259 newborns (0.19%) (Table 1). These cut-offs represented the population 99.9th and 99.5th %iles respectively. The second-tier LC-MS/MS assay separated out an interfering substance and yielded GAA values below 3.5 μM for all but 3 specimens (GAA = 3.5, 3.9 and 6.8 μM). Molecular analysis did not identify any variants in *GAMT* in these 3 DNA samples, thus all were deemed negative screens, and no follow-up was initiated as per the study protocol (Fig. 3).

3.3. Interfering compound

Although the de-identification process for this study limits available information on the samples with elevated GAA, some trends can be discerned. Repeat specimens (second samples) are requested on all infants <1500 g due to the risk of false negative congenital hypothyroidism screens in very low birth weight and premature infants [16]. Repeat specimens account for 4% of total samples received over the study period but represented 44% of the samples flagged for second-tier testing due to an elevated GAA (Table 1). The mean birth weight for these repeat samples (2212 g) was also significantly lower than the population average (3372 g) showing enrichment for low birth weight infants. Importantly, second-tier testing resulted in normal GAA values for the vast majority of these samples due to separation of an interfering peak. It appears that low birth weight infants are more likely to have falsely elevated GAA on first-tier testing due to this interference. This phenomenon only occurs on repeat samples, not the initial newborn screens. It is hypothesized that this interference is likely an exogenous compound resulting from therapeutic interventions implemented for these high-risk infants, although a delayed rise in an endogenous compound cannot be excluded. Similar conclusions were reached by Pitt et al. (2014) [10]. Unfortunately, spectral library searching using the full MS/MS fragmentation pattern for this peak has failed to identify a likely candidate compound.

4. Discussion

An integrated three-tier approach to GAMT deficiency newborn screening has shown highly favourable test performance with zero false positive results after 3 years of screening on >135,000 routine newborn screening specimens. Both the first and second-tier tests have been integrated into existing laboratory assays minimizing the incremental 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), were successfully corrected by the second-tier LC-MS/MS assay. Given that this compound was identified in repeat (but not initial) specimens, it is likely of exogenous origin as has been postulated previously [10].

The true incidence of GAMT deficiency remains unclear. Although a founder affect has led to an increased incidence of 1/60,000 in Portugal, the disorder appears otherwise rare world wide. *GAMT* mutation carrier frequencies have been estimated in two small molecular screening studies at 1:250 in the Netherlands and 1:1475 in British Columbia but both studies suffered from low case numbers and these estimates have very wide confidence intervals [3,11]. Desroches et al. utilized functional and *in silico* analyses of variants reported in public databases to reach *GAMT* mutation carrier estimates of 1:812 (functional validation) and 1:372 (*in silico* analysis) but as noted by the authors, the methodologies utilized likely under and over-estimated these frequencies respectively [4]. As previously noted, when all GAMT newborn screening pilots are compiled (including this study), over 1 million infants have been screened world-wide without the identification of a single affected infant [3,10]. While this suggests that GAMT deficiency is a very rare disease, it remains possible that affected individuals may have been missed by this and other pilot studies given the minimal data available on GAA levels in affected newborns.

The decision to include a disorder in a newborn screening panel requires a thorough evaluation of the benefits and costs of screening, as originally described by Wilson and Junger [17]. Although these original criteria have been modified over time and with the introduction of multi-analyte screening technologies, aspects of disease significance, therapeutic impact, test performance and financial costs remain central to the process of evaluating a candidate disorder. GAMT deficiency is an ultra-rare disorder without a clear population incidence, but comprehensive review of clinically ascertained cases confirms that developmental outcomes are universally poor with post-symptomatic initiation of therapy [7]. In stark contrast, the 4 individuals identified and treated from birth onwards due to a prior family history have had excellent neurodevelopmental outcomes [3,7]. From a test performance and cost perspective, two-tier GAA biochemical screening can be integrated into existing assays with negligible impact on reagent and labour costs. The further inclusion of the third-tier dried bloodspot *GAMT* molecular analysis step has been able to reduce the false positive rate to zero in this pilot, further minimizing the potential harms and downstream system costs of screening. Given that only 3 samples required sequencing in 135,000 infants screened, this added step did little to the overall cost of the screening program.

Table 1
GAMT screening analytical results.

	Newborn population	Above first-tier cutoff[a] (6 μM/3.5 μM)	Above second-tier cutoff[a] (6 μM/3.5 μM)	Historical GAMT cases [10]
Total sample number	135,372	259	3	
Initial screens	129,957	145	3	
Repeat screens	5415	114		
First-tier GAA (μM)				
Mean (SD)	1.54 (0.45)	7.14 (5.37)	6.1, 7.9, 4.8	10.7, 9.1
Range	0.93–2.49 (2.5th–97.5th)	3.51–42.0 (min-max)		
Second-tier GAA (μM)				
Mean (SD)		1.45 (0.74)	6.8, 3.5, 3.9	N/A
Range		0.3–6.8 (min-max)		

[a] The screening cutoff for both first and second-tier GAA assays was set at the 99.9th%ile of the population range (6 μM) for the first 18 months, and lowered to the 99.5th%ile (3.5 μM) for the final 18 months.

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4.1. Conclusions

Despite the apparent low incidence, GAMT deficiency remains an excellent candidate for routine newborn screening in laboratories with existing capacity for second-tier MS/MS testing and/or bloodspot sequencing capacity given the promising outcomes seen with early therapeutic intervention. These conclusions are reinforced by the recent recommendations of the Health Council of the Netherlands to include GAMT deficiency to their neonatal screening program and the initiation of population-wide screening in Utah in July 2015. [12,18].

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