Galactose-1-Phosphate Uridyltransferase Activities in Different Genotypes: A Retrospective Analysis of 927 Samples

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Background: Classic galactosemia is an inherited disorder of galactose metabolism caused by the impaired activity of galactose-1-phosphate uridyltransferase (GALT). Untreated galactosemia is life-threatening; however, early dietary intervention prevents mortality and reduces morbidity associated with this disease. The diagnosis of galactosemia includes the measurement of GALT activity in red blood cells (RBC) and *GALT* gene analysis. In this study, we evaluate GALT activity in different genotypes using the results of combined biochemical and molecular testing in 927 samples.

Methods: GALT activity in RBC was measured by LC-MS/MS. The analysis of the *GALT* gene was performed by targeted gene analysis and/or full gene sequencing. Samples were assigned based on the presence of pathogenic (G) or Duarte 2 (D) variants, or their absence (Neg), to G/G, D/G, G/Neg, D/D, D/Neg, and Neg/Neg genotypes. Finite mixture models were applied to investigate distributions of GALT activities in these genotypes. The reference ranges were determined using the central 95% of values of GALT activities.

Results: The ranges of GALT activity in G/G, D/G, G/Neg, D/D, D/Neg, and Neg/Neg genotypes are 0.0 to 0.7 $\mu mol \cdot h^{-1}$ gHb^{-1}, 3.1 to 7.8 $\mu mol \cdot h^{-1}$ gHb^{-1}, 6.5 to 16.2 $\mu mol \cdot h^{-1}$ gHb^{-1}, 6.4 to 16.5 $\mu mol \cdot h^{-1}$ gHb^{-1}, 12.0 to 24.0 $\mu mol \cdot h^{-1}$ gHb^{-1}, and 19.4 to 33.4 $\mu mol \cdot h^{-1}$ gHb^{-1}, respectively.

Conclusions: The GALT activity ranges established in this study are in agreement with the expected impact of the genotype on the enzymatic activity. Molecular findings should be interpreted in view of biochemical results to confirm genotype–phenotype correlation.

IMPACT STATEMENT

This study describes results from 927 samples submitted to our laboratory for galactose-1-phosphate uridyltransferase (GALT) enzyme activity testing by LC-MS/MS in combination with molecular analysis of the *GALT* gene. Using statistical algorithms, we established the ranges of GALT activities for different genotypes, including classic and Duarte variant galactosemia, carriers for classic galactosemia and unaffected control. This study expands our understanding of genotype–phenotype correlation in galactosemia, which is important for interpretation of the results of diagnostic testing.

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Galactose-1-phosphate uridyltransferase (GALT; EC 2.7.7.12)[4] is an enzyme in the Leloir pathway that catalyzes the reaction between galactose-1-phosphate and UDP-glucose to produce glucose-1-phosphate and UDP-galactose. The impaired activity of this enzyme causes classic galactosemia, an inherited disorder of galactose metabolism (Online Mendelian Inheritance in Man 230400) with an incidence between 1:14 000 and 1:80 000 (mean, 1:41 219) (*1*). The disorder acutely presents in the newborn period with jaundice, hepatomegaly, liver failure, coagulopathy, and *Escherichia coli* sepsis (*2*). Early diagnosis and dietary lactose restriction are imperative to prevent these life-threatening complications; therefore, galactosemia is included in newborn screening panels in the US and many other countries.

Confirmatory testing of a positive newborn screen for galactosemia includes the measurement of GALT activity in red blood cells (RBC) and molecular testing of the *GALT*[5] gene by targeted DNA analysis or full gene sequencing (*5*). At present, >300 *GALT* variants have been described (GALT database: http://www.arup.utah.edu/database/GALT/GALT_display.php). The most frequently encountered variant is Duarte 2 (p.N314D; allele frequency 0.09201), which is characterized by 50% of normal GALT activity (*3*–*5*). Among pathogenic variants, p.Q188R, p.K285N, and p.L195P are common in whites (*6*–*10*), whereas p.S135L and IVS2-2A>G variants are frequently reported in patients of African and Hispanic origins, respectively (*11*, *12*). Patients with the *S135L/S135L* genotype retain some residual GALT activity in liver and other tissues (*2*, *11*, *13*) but still require dietary galactose restriction to prevent long-term complications. Individuals with Duarte variant galactosemia (compound heterozygosity for Duarte 2 and a pathogenic variant) have 15% to 25% of normal GALT activity in RBC, and their clinical outcomes are good regardless of dietary restriction (*4*, *14*).

The evaluation of GALT activity in RBC is central in a diagnostic workup for galactosemia. Until recently, clinical laboratories used fluorescent and radioactive assays for the measurement of the enzyme activity. In addition to being labor intensive and inconvenient, these assays also lack sufficient sensitivity at the low range of enzyme activities, which is important for accurate determination of residual GALT activity in galactosemia patients (*15*–*17*). Introduction of the new LC-MS/MS method for the measurement of RBC GALT activity helped to overcome these disadvantages (*15*, *16*, *18*). The method relies on the quantification of the stable isotope-labeled product of the endogenous GALT reaction, and its analytical characteristics have been described in detail (*15*, *16*, *18*). These studies reported GALT activities in several affected and unaffected patients; however, the sample cohort used to assess the genotype–phenotype correlation was limited.

Here, we analyzed data from 927 samples submitted to our laboratory for GALT activity testing and DNA analysis of the *GALT* gene and established ranges for GALT activity in different genotypes, including classic and Duarte variant galactosemia, as well as in unaffected controls.

MATERIALS AND METHODS

All authors were compliant and followed the ethical guidelines according to the requirements of the Institutional Review Board of the University of Utah.

Molecular testing

The analysis of the *GALT* gene was performed by (*a*) targeted gene analysis (877 samples), (*b*)

[4] **Nonstandard abbreviations:** GALT, galactose-1-phosphate uridyltransferase; RBC, red blood cells; G, pathogenic variant; D, Duarte 2 variant; AIC, Akaike information criterion; LA, Duarte 1 variant.
[5] **Human gene:** GALT, galactose-1-phosphate uridylyltransferase.

full gene sequencing (31 samples), or (*c*) both methods (19 samples), in which case only results of full gene sequencing were used for genotype–biochemical phenotype correlation. Genomic DNA was extracted from peripheral blood white blood cells via automated MagNa Pure LC DNA Isolation ZKit (Roche) following the manufacturer's procedure. Targeted analysis of 9 variants in the *GALT* gene (IVS2-2A>G, S135L, T138M, L195P, K285N, Q188R, Y209C, N314D, and L218L) was performed as described (*19*, *20*). For full *GALT* gene analysis, the coding regions (including exon/intron boundaries with ±30 bp into the intronic sequences) and the 5'-untranslated region for Duarte 2 promoter region variant c.-119_-116delGTCA were amplified and sequenced by capillary electrophoresis on the ABI PRISM 3730 Genetics Analyzer (Applied Biosystems) following manufacturer's instruction. Sequence data were aligned to GenBank's human GALT sequence (NC_000009.12, NM_000155.2) and analyzed using Mutation Surveyor 5.0 (Softgenetics).

Based on the presence of pathogenic (G) or Duarte 2 (D) variant in the *GALT* gene, or their absence (Neg), samples were assigned to G/G, D/G, G/Neg, D/D, D/Neg, and Neg/Neg genotypes. For samples submitted for targeted gene analysis, "Neg" genotype indicated only the absence of the variants included in the panel.

Biochemical testing

The measurement of GALT activity in RBC was performed by LC-MS/MS according to published procedures (*16*). An API 4000 tandem mass spectrometer (AB Sciex) equipped with Shimadzu liquid chromatography units (Shimadzu North America) was used in this study. Chromatographic separation was achieved on a Waters XBridge BEH amide column (3.5 µm, 2.1 mm × 100 mm) using a linear gradient of mobile phase A (5 mmol/L ammonium formate in acetonitrile/water, 90:10 v/v) and mobile phase B (5 mmol/L ammonium formate in acetonitrile/water, 50:50 v/v) with flow rate 0.4 mL/min as follows: 0 min, 15% B; 3 min, 100% B; 5.4 min, 100% B; 6.5 min, 15% B; 14.5 min, 15% B. The total run time, injection to injection, was 14.5 min.

Statistical analysis

Finite mixture models were applied to investigate distributions of GALT enzymatic activities in D/D, D/Neg, and Neg/Neg genotypes using an expectation–maximization algorithm (Fig. 1) (*21*–*24*). This method provides estimates of the means (μ_1, μ_2), SDs (σ_1, σ_2), and mixture proportion (p) that are most likely given the data. Computations were performed using the mixtools R package (*22*). Likelihoods of mixed vs single component distributions were assessed for each of these distributions using the Akaike information criterion (AIC), with lower AIC scores indicating a better fit. The reference ranges were determined by using the central 95% of values of GALT activities in the reference intervals R package (*25*). Parametric estimates were generated for G/G, D/G, G/Neg, D/D, and D/Neg genotypes, and nonparametric estimates were done using bootstrap resampling for the Neg/Neg genotype (*13*).

All calculations were performed using R version 3.3.3 (2017–03-06) (Copyright 2017 The R Foundation for Statistical Computing).

RESULTS

Targeted and full gene analysis of the *GALT* gene

We analyzed GALT enzyme activity in combination with mutational analysis of the *GALT* gene in 927 samples. The majority of samples were from patients <1 year of age, suggesting that testing was most likely owing to follow-up of abnormal newborn screening results for galactosemia. Targeted DNA analysis (GALT panel), which includes 7 common pathogenic variants (p.Q188R, p.S135L, p.K285N, p.T138M, p.L195P, p.Y209C, IVS2-2 A>G) and 2 Duarte variants (p.N314D and p.L218L), was

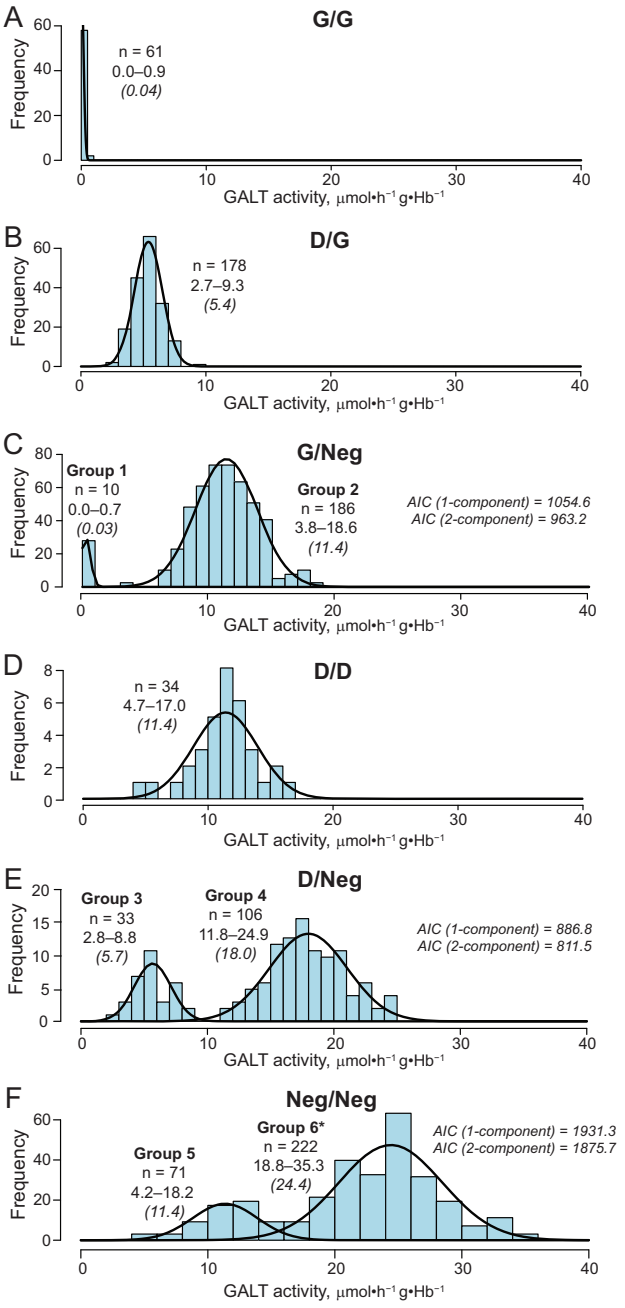

Fig. 1. Distribution of GALT activities in the samples submitted for molecular analysis of the *GALT* gene. A bimodal distribution of activities explains discrepancies between biochemical and molecular findings in group 1 (G/Neg), group 3 (D/Neg), and group 5 (Neg/Neg) and reflects the presence of *GALT* variants not detected by targeted gene analysis. The AIC scores were used to estimate the quality of statistical models. A 2-component solution provided better (lower) AIC scores for G/Neg, D/Neg, and Neg/Neg activities. n, number of samples in each group; minimum and maximum GALT activities are shown below with mean values in parenthesis. *Only Neg/Neg samples with GALT activities above those observed in G/Neg group 2 samples (>18.6 µmol·h^{-1} gHb^{-1}; C) were used in calculation of the Neg/Neg range (see text).

Table 1. Ranges of GALT activities in different genotypes.[a]

Genotype	Total number of samples	GALT activity range (mean), μmol·h^{-1} gHb^{-1}	Percentage of normal activity[b]
G/G	71	0.0–0.7[c] (0.07)	<1
D/G	211	3.1–7.8 (5.5)	22.1
G/Neg	257	6.5–16.2 (11.7)	47.0
D/D	34	6.4–16.5 (11.4)	45.8
D/Neg	106	12.0–24.0 (18.0)	72.3
Neg/Neg	222	19.4–33.4 (24.9)	100

[a] Calculated using the central 95% of values of GALT activities.
[b] Mean activity of genotype relative to mean Neg/Neg activity, in %.
[c] Nonzero GALT activities, 0.3–0.9 μmol·h^{-1} gHb^{-1}, were detected in 3 samples heterozygous for pQ188R and p.S135L by targeted gene analysis, and 3 samples with Q188R/R258C, Q188R/T138M, and V151A/G214R genotypes by full gene sequencing.

performed in 877 samples (approximately 95% of all samples). Thirty-one samples were analyzed by full gene sequencing. In addition, 19 samples were submitted for targeted gene analysis and, subsequently, full gene sequencing, bringing the total number of samples analyzed by full gene sequencing to 50.

The most common variants detected were p.Q188R (21.4%), p.N314D (20.7%), p.S135L (3.2%), p.K285N (1.2%), p.L195P (0.6%), IVS2-2A>G (0.5%), p.T138M (0.2%), and p.Y209C (0.2%), in agreement with previous studies (*6–8*, *10*). Overall, 31 rare pathogenic variants (frequency of each <0.2%) were detected by full gene sequencing (see http://www.arup.utah.edu/database/GALT/GALT_display.php for the complete list of variants identified by ARUP Laboratories). The frequency of negative alleles was 49.3% and 27.0% with targeted mutation panel and full gene sequencing, respectively.

The results of *GALT* gene analysis were used to assign samples to G/G, D/G, G/Neg, D/D, D/Neg, and Neg/Neg genotypes based on the presence of pathogenic (G) or Duarte 2 (D) variant in the *GALT* gene, or their absence (Neg). The total number of samples (n = 26) in which the p.L218L variant (LA) was detected associated with a pathogenic variant (LA/G), the Duarte 2 variant (LA/D), or by itself (LA/Neg; LA/LA) was too small to apply the statistical analysis used to determine GALT activities, and those samples were excluded from the analysis. The detection of pathogenic variants in the majority of samples (approximately 95%) was limited to the 9 variants included in the panel; therefore, in a subset of the samples, the enzyme activity was lower than expected based on the results of targeted DNA analysis, indicating the presence of other *GALT* variants not detected by the testing (see below). Variants in regulatory or deep intronic regions, as well as large deletions/duplications, were also not identified by full gene sequencing.

GALT activity in different genotypes

The enzyme activity values obtained for the genotypes detected by DNA testing (targeted and full gene sequencing) were subjected to the statistical analysis previously described (see Materials and Methods section). All values associated with each genotype were analyzed.

The distribution of GALT activities in G/G, D/G, G/Neg, D/D, D/Neg, and Neg/Neg samples is shown in Fig. 1. As expected, the majority of G/G patients (patients with biallelic pathogenic variants) had undetectable enzymatic activities. Enzyme activities associated with Duarte variant galactosemia (D/G) genotype, in which the pathogenic variant (G) was identified, were in the range of 3.1 to 7.8 μmol·h^{-1} gHb^{-1} (Table 1) also in agreement with previous studies (*4*, *20*). Notably, approximately 16% of samples associated with

G/Neg, D/Neg, and Neg/Neg genotypes showed genotype–biochemical phenotype discrepancies, reflecting the presence in those samples of pathogenic variants not identified by targeted gene analysis. GALT activities in G/Neg samples were distributed into 2 distinct groups: group 1 with enzyme activity in the G/G range (<0.7 μmol·h^{-1} gHb^{-1}), in agreement with classic galactosemia, and group 2 with a mean activity twice that of D/G (11.4 μmol·h^{-1} gHb^{-1}), concordant with carrier status (Fig. 1C). Similarly, in samples with the apparent D/Neg genotype (groups 3 and 4; Fig. 1E) and Neg/Neg genotype (groups 5 and 6; Fig. 1F), a bimodal distribution of enzyme activities was observed (Fig. 1). The presence of 2 distinct populations within G/Neg, D/Neg, and Neg/Neg activities was confirmed using finite mixture models (*21*). In these cases, a 2-component solution provided better (lower) AIC scores.

The bimodal distribution of G/Neg, D/Neg, and Neg/Neg activities (Fig. 1) reflects the presence of a causative allele in the *GALT* gene that is not detected by targeted DNA analysis. This assumption is supported by a significant overlap in enzyme activities between G/Neg group 1 and G/G (0.0–0.7 vs 0.0–0.9 μmol·h^{-1} gHb^{-1}), D/Neg group 3 and D/G (2.8–8.8 vs 2.7–9.3 μmol·h^{-1} gHb^{-1}), and G/Neg group 2 and Neg/Neg group 5 (3.8–18.6 vs 4.2–18.2 μmol·h^{-1} gHb^{-1}) (Fig. 1). Further, the presence of pathogenic variants in the *GALT* gene that were not included in the panel was confirmed in 19 samples, which were first submitted for targeted gene analysis and then for full gene sequencing to resolve discrepancies between molecular and biochemical findings. Targeted gene analysis detected only 1 pathogenic variant in 9 samples with markedly reduced GALT activity, <0.7 μmol·h^{-1} gHb^{-1}, and 1 Duarte 2 variant in 10 samples with GALT activity in the range of 3.5 to 7.4 μmol·h^{-1} gHb^{-1}. All samples were found to have a second pathogenic GALT variant by full gene sequencing (e.g., p.H321Y, p.Q212X, p.E58K, p.R148W, p.R231C), confirming that their GALT activities, in fact, were consistent with G/G and D/G genotypes, respectively.

Based on the results of the statistical analysis, which were confirmed by full gene sequencing in a subset of the samples (total n = 19, see above), we reassigned group 1 and group 3 activities to G/G and D/G genotypes, respectively, and then combined results were used (Fig. 1: G/G + group 1, D/G + group 3) to establish G/G and D/G enzymatic ranges. Because of partial overlap in the distributions of group 5 and 6 activities (Fig. 1F), only Neg/Neg samples with GALT activities above the highest activity observed in G/Neg samples in group 2 (>18.6 μmol·h^{-1} gHb^{-1}; Fig. 1C) were included in the Neg/Neg range (n = 222). The remaining Neg/Neg samples with GALT activities ≤18.6 μmol·h^{-1} gHb^{-1} were combined with G/Neg samples in group 2 to calculate the range of G/Neg activities (total n = 257). Final ranges for all genotypes were established using the central 95% of values of GALT activities (Table 1).

The enzymatic activities in G/G, D/G, G/Neg, D/D, and D/Neg genotypes varied from <1% to approximately 72% of Neg/Neg activity in accordance with the expected genotype impact on GALT activity (Table 1). There was a significant overlap between heterozygotes for pathogenic variants (G/Neg) and homozygotes for Duarte 2 variant (D/D) both associated with 50% of the normal activity. The activities in approximately 25% of G/Neg and D/D samples also overlapped with GALT activities in carriers of the Duarte 2 allele (D/Neg) (Table 1), showing that biochemical testing alone is not sufficient to determine carrier status for classic galactosemia. The reference range, corresponding to Neg/Neg activities, was comparable with the range previously reported in other studies (*16*).

GALT activities in 46 of 50 samples analyzed by full gene sequencing (see Materials and Methods section) were within the established ranges (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.jalm.org/content/vol3/issue2). However, 4

samples showed genotype–biochemical phenotype discrepancies. One G/G sample homozygous for the silent variant p.N288N had GALT activity higher than usually observed in G/G genotypes, at 1.8 μmol·h^{-1} gHb^{-1}, but significantly lower than in D/G or G/Neg genotypes. This variant has been suggested to activate a cryptic exonic acceptor splice site (*8*). In addition, GALT activities in 3 Neg/Neg samples were lower than expected—4.2, 12.2, and 14.7 μmol·h^{-1} gHb^{-1} (all included in group 5; Fig. 1)—likely reflecting the presence of a variant in the regulatory or deep intronic region, and/or a deletion in the *GALT* gene not detected by the test.

DISCUSSION

The goal of this study was to establish ranges of GALT activities in different genotypes based on retrospective analysis of biochemical and molecular results in 927 samples received by our laboratory since 2013. GALT activity in RBC was measured by an established LC-MS/MS method (*15*), whereas analysis of the *GALT* gene was performed by targeted gene analysis limited to the 9 most common variants (877 samples) and/or full gene sequencing (50 samples; see Material and Methods section).

The results of biochemical and molecular testing showed a normal distribution of GALT activities in G/G, D/G, and D/D samples (Fig. 1), and the relationship between genotype and biochemical phenotype in these samples was straightforward. Interestingly, among the samples with GALT activities in the classic galactosemia range, 6 samples showed detectable GALT activity, 0.3 to 0.9 μmol·h^{-1} gHb^{-1} (Table 1). Three samples were heterozygous for p.Q188R and p.S135L by targeted gene analysis (G/Neg), suggesting the presence of a second pathogenic allele. Unfortunately, these samples were not available for further molecular testing. Three other samples analyzed by full gene sequencing were found to have *Q188R/R258C*, *Q188R/T138M*, and *V151A/ G214R* genotypes (see Table 1 in the online Data Supplement). Additional investigations would be necessary to determine whether nonzero GALT activities observed in these rare genotypes reflected their true residual enzymatic activity or analytical artifacts.

In contrast to the aforementioned samples, G/Neg, D/Neg, and Neg/Neg genotypes were associated with activities showing a clear bimodal distribution consistent with the presence of rare pathogenic variants not detected by targeted gene analysis. Ideally, these genotype–biochemical phenotype discrepancies could have been resolved by sequencing and, if necessary, deletion/duplication analysis of the *GALT* gene of all 114 samples with lower than expected enzymatic activities (samples in groups 1, 3, and 5, combined; Fig. 1), as it was done for the other 19 samples described in the Results section. However, this was not feasible because of the retrospective nature of the study. To help overcome this limitation, we used statistical algorithms to reassign enzyme activities to the appropriate genotypes, which, in combination with the large number of samples, were the main strengths of this study. The GALT activity ranges established are in agreement with the expected impact of the genotype on the enzymatic activity, demonstrating the validity of this approach (Table 1). GALT activities in 46 of 50 samples analyzed by full gene sequencing also confirmed the validity of this approach. The presence of a variant in a noncoding region and/or a deletion in the *GALT* gene may explain the discrepancies in 4 other samples.

The main advantages of targeted mutational analysis are relatively low cost and relatively rapid turnaround time. This study shows also that molecular findings should be interpreted in view of biochemical results to confirm genotype–biochemical phenotype correlation. Expanding targeted analysis to other pathogenic variants, for example, M142K and R333W both with 4.12e^{-05} allele frequencies (*3*, *6*, *8*, *10*), would improve genotype–biochemical phenotype correlation and decrease the number of samples

requiring full gene sequencing. Introduction of next-generation sequencing panels into molecular genetics laboratory practice will eventually replace traditional single-gene analysis. However, even with a significantly higher rate of variant detection expected from next-generation sequencing technology, biochemical testing will remain a central part of the diagnostic workup for patients with genetically heterogeneous metabolic disorders, such as galactosemia, and with disorders for which strong genotype–phenotype association cannot be established.

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