

# Lymphocyte Galactocerebrosidase Activity by LC-MS/MS for Post-Newborn Screening Evaluation of Krabbe Disease

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**BACKGROUND:** Deficiency of the lysosomal enzyme galactosylcerebrosidase (GALC) causes Krabbe disease. Newborn screening for Krabbe disease is ongoing, but improved methods for follow-up analysis of screen-positive babies are needed to better advise families and to optimize treatment. We report a new assay for the enzymatic activity of GALC in lymphocytes.

**METHODS:** T lymphocytes were isolated from venous blood by magnetic bead technology. The assay used a close structural analog of the natural substrate and LC-MS/MS to quantify the amount of product with the aid of a chemically identical internal standard.

**RESULTS:** The analytical range of the assay (ratio of assay response for the QC high standard to that from all non-enzymatic-dependent processes) was 20-fold greater than that for the conventional radiometric GALC assay. The LC-MS/MS could distinguish cells that were null in GALC from those that contained traces of active enzyme (down to 0.3% of normal). There was a good correlation between the level of residual GALC activity in lymphocytes and the severity of Krabbe disease.

**CONCLUSIONS:** The new assay can measure small amounts of residual GALC activity in leukocytes with high accuracy compared to previous assays and can contribute, along with genotyping, biomarker analysis, and neurological imaging, a better plan for post-newborn screening follow-up for Krabbe disease.

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Krabbe disease (globoid cell leukodystrophy) is an often-fatal lysosomal storage disease caused by the deficiency of the enzyme galactocerebrosidase (GALC;<sup>8</sup> EC 3.2.1.46), resulting in the loss of the myelin sheath in the central nervous system (1, 2). Interest in newborn screening for Krabbe disease has escalated owing to the demonstration that bone marrow transplantation in children before the onset of severe symptoms is effective at suppressing the onset of the disease (3). State newborn screening laboratories in New York (4), Missouri, Ohio, and Kentucky now carry out newborn screening for Krabbe disease, and Illinois and New Jersey are expected to start soon.

Newborn screening for Krabbe disease is performed by direct measurement of GALC enzymatic activity in dried blood spots either by tandem mass spectrometry (MS/MS) or by fluorometry (5). Over the past approximately 10 years, about 3.3 million newborns have been screened for Krabbe disease in New York by MS/MS. Of these tested newborns, 5 were confirmed by clinical exam follow-up to have early infantile Krabbe disease, and 12 additional patients were found to be at high risk for the development of Krabbe disease but have so far remained asymptomatic (4). The high-risk designation applies to newborns that display the lowest bracket of GALC enzymatic activity determined using a radiometric assay in lymphocytes ( $\leq 0.15$  nmol product/h/mg protein) (6). All of these high-risk patients also have 2 Krabbe disease-associated alleles. Based on these data, it is generally thought that the level of residual GALC activity in leukocytes is not sufficient to explain the severity of Krabbe disease and to differentiate between symptomatic and asymptomatic patients. The value of the radiometric

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<sup>8</sup> Nonstandard abbreviations: GALC, galactocerebrosidase; MS/MS, tandem mass spectrometry; GALC-S, galactosylceramide with a 7-carbon heptanoyl chain; GALC-IS, GALC internal standard; GALC-P, GALC product; GLB1, human  $\beta$ -galactosidase-1; MRM, multiple reaction monitoring; LC, liquid chromatography; dpm, disintegration per minute; EIKD, early-infantile Krabbe disease; LOKD, late-onset Krabbe disease.

GALC assay is in the confirmation of the diagnosis of Krabbe disease in already symptomatic patients.

Patients who are null in GALC because of 2 severe mutations, including a large 30-kb deletion that removes several exons of the gene (7), are thought to develop early infantile Krabbe disease, strongly suggesting that there is not a second enzyme that can compensate for the loss of GALC enzymatic activity. We hypothesize that the severity of Krabbe disease, including the age of onset of symptoms, is dictated mainly by the degree of residual GALC activity. There is the possibility that the currently used radiometric assay for GALC is insufficiently accurate to measure small differences in residual enzymatic activity when the activity is close to zero. To study this further, we have focused our efforts on the development of a new assay of GALC enzymatic activity that has much higher resolution than the current radiometric assay so that small differences in GALC activity close to zero can be measured in a statistically confident manner. Here, we describe a new GALC assay based on LC-MS/MS that accomplishes this goal.

## Methods

T lymphocytes were isolated from venous blood by magnetic bead technology. LC-MS/MS GALC assays were carried out with a close structural analog (galactosylceramide, with a heptanoyl fatty acyl chain) of the natural substrate. The internal standard is chemically identical to the substrate but differentiated by deuterium substitution. Standard operating procedures for all methods in this article are provided in the Supplemental Material file that accompanies the online version of this article at <http://www.clinchem.org/content/vol63/issue8>.

## Results and Discussion

### GALC ASSAY REAGENTS, CELL SOURCE, AND BUFFER

Our original MS/MS assay of GALC was based on the substrate galactosylceramide with an octanoyl attached to the sphingosine backbone (8). We now prefer galactosylceramide with a 7-carbon heptanoyl chain (GALC-S) because it dissolves more rapidly in buffer than the original substrate. The internal standard (GALC-IS) is chemically identical to the GALC product (GALC-P) but contains a terminal CD<sub>2</sub>CD<sub>3</sub> in the heptanoyl chain. The use of a chemically identical internal standard accounts for all losses of GALC-P and for ionization suppression during MS/MS.

For initial studies, we decided to isolate a single type of leukocyte from whole blood, namely T lymphocytes, by using magnetic beads containing antibody to the cell surface CD3 protein. Although there have been no reports of the relative amounts of GALC in different types of leukocytes, we were concerned that if there were dif-

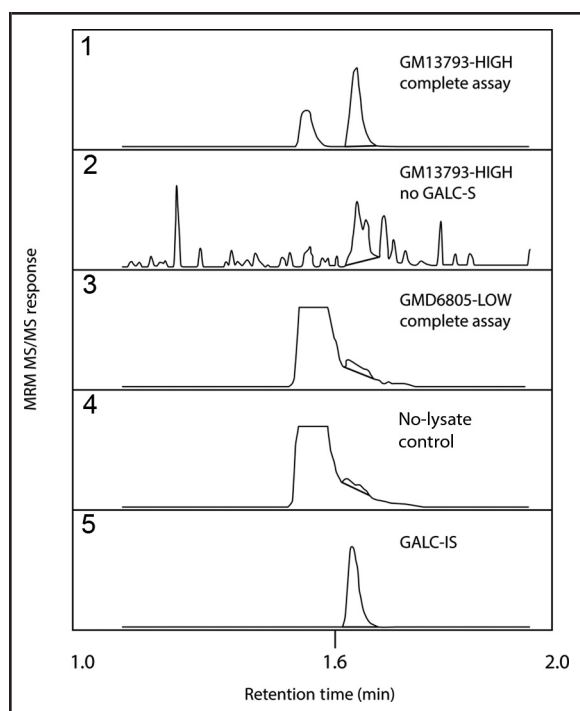
ferences, the measured amount of GALC enzymatic activity per milligram of cellular protein would be a function of differential cell counts. It would also be a function of differential loss of white cell types during the 1–2 days required for shipment of blood to the biochemical genetics laboratory. Recovery of T lymphocytes using the magnetic bead technology was >80%.

The previously published buffer for in vitro assay of GALC was citrate-phosphate, pH 4.2 (6). We measured the activity of recombinant human GALC in this buffer and also in 0.2 mol/L sodium acetate, pH 4.0. Both buffers also contained sodium taurocholate and sodium oleate, which activate GALC (6). For reasons described below, we also tested the activity of recombinant human  $\beta$ -galactosidase 1 (GLB1). In sodium acetate buffer, the MS/MS ion counts for enzymatic product obtained using lymphoblast cells from a Pompe disease patient (Coriell Biorepository, GM13793, our functional GALC control cell line) divided by the ion counts for the internal standard were 2.728 for GALC and 0.032 for GLB1. In citrate phosphate buffer, the ratios were 20.498 for GALC and 0.004 for GLB1. Thus, we preferred citrate phosphate buffer and used this for all subsequent studies.

### LC-MS/MS ASSAY OF GALC

Before studies with blood cells we measured GALC in lysates of LCL (lymphoblastic cell line) lymphoblasts from the Coriell Cell Repository. The Pompe cell line GM13793 constituted our HIGH QC sample for the study (GM13793-HIGH). The second cell line, GM06805 (GM06805-LOW) was from a patient who was assumed to be completely devoid of functional GALC enzyme because approximately 30% of the exons had been deleted from both copies of the galactosylceramidase (*GALC*) gene [homozygotes develop early infantile Krabbe disease (7)].

Lysates from these cells were used to perform assays containing 2  $\mu$ g of cell protein in an assay cocktail consisting of 150  $\mu$ mol/L GALC-S and 5  $\mu$ mol/L GALC-IS in citrate-phosphate buffer with sodium oleate and sodium taurocholate, pH 4.2. The LC-MS/MS ion traces for GALC-P are shown in Fig. 1. Table 1 contains the ion counts for the GALC-P and GALC-IS obtained by integrating the multiple reaction monitoring (MRM) ion peaks vs the liquid chromatography (LC) retention time profile shown in Fig. 1. The complete assay, in which GM13793-HIGH lysate was incubated with GALC-S, is shown in the top panel of Fig. 1. The peak at approximately 1.67 min was due to GALC-generated GALC-P as it comigrated with the chemically identical GALC-IS (bottom panel). The peak in the top panel eluting at approximately 1.57 min is GALC-P produced by nonenzymatic cleavage of GALC-S in the heated electrospray ionization source of the MS/MS instrument as it comigrates with GALC-S (not shown). This in-source cleavage



**Fig. 1.** LC-MS/MS ion chromatograms.

Complete assay with GALC-S incubated with GM13793-HIGH cell lysate (panel 1); GM13793-HIGH lysate incubated in the absence of GALC-S (panel 2); GM06805-LOW lysate incubated with GALC-S (panel 3); and GALC-S incubated in buffer without cell lysate (panel 4).

Panels 1–4 show the MRM ion chromatograms for GALC-P; panel 5 is for GALC-IS.

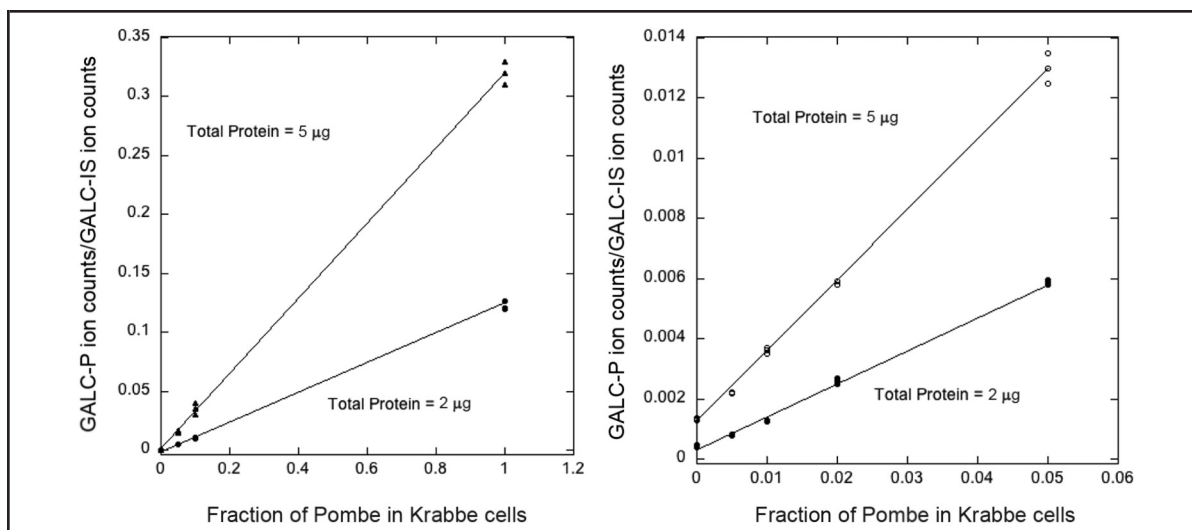
was of no consequence because the 2 GALC-P peaks were baseline separated and only the 1.67 min was integrated to determine the GALC activity.

The first control assay was the cell lysate (GM13793-HIGH) incubated without GALC-S (Fig. 1, panel 2), which showed essentially no GALC-P signal (0.007% of the activity seen with GM13793-HIGH in Fig. 1, panel 1, and Table 1). This indicated that the lysate did not contain compounds that were isobaric with GALC-P and that eluted at the same LC retention time. The second control was GALC-S incubated in buffer without cell lysate (Fig. 1, panel 4), which had a GALC-P signal that was only 0.07% of the signal seen with cell lysate (Table 1), showing that GALC-S contained essentially no GALC-P as an impurity and that GALC-S underwent essentially no nonenzymatic breakdown to GALC-P. Fig. 1, panel 3, shows the assay with GM06805-LOW incubated with GALC-S. The signal was approximately 10-fold higher than that obtained in the no-lysate and represented 0.4% of the signal seen with GM13793-HIGH (Table 1). This small increase in assay response was attributed to one or more non-GALC enzymes present in the cell lysate that were capable of generating GALC-P from GALC-S. One candidate is GLB1, since this enzyme cleaves terminal  $\beta$ -linked galactosyl residues from gangliosides. GLB1 deficiency causes 2 lysosomal storage disorders unrelated to Krabbe disease, namely GM<sub>1</sub>-gangliosidosis and Morquio B syndrome. For the assay with GM13793-HIGH the GALC-P assay response was 148 719 ion counts (mean of triplicate runs, Table 1), and thus 150-fold higher than the value seen with the GALC-null cells, GM06805-LOW.

**Table 1.** LC-MS/MS GALC assay results.<sup>a</sup>

Assay	GALC-P ion counts	GALC-IS ion counts	GALC activity, nmol/h/mg, mean $\pm$ SD
GM13793-HIGH lysate + GALC-S	143 518	1 246 449	0.5837 $\pm$ 0.0406
	147 838	1 171 068	
	154 801	1 171 068	
No-lysate control	89	1 231 481	0.000364 $\pm$ 0.000119
	64	1 150 753	
	122	1 156 488	
GM13793-HIGH lysate minus GALC-S	10	563 384	7.25 $\times$ 10 <sup>-5</sup> $\pm$ 0.979 $\times$ 10 <sup>-5</sup>
	8	586 144	
	8	532 992	
GM06805-LOW + GALC-S	1 129	1 352 893	0.00371 $\pm$ 0.00038
	1 110	1 316 255	
	880	1 260 690	

<sup>a</sup> Instrument noise was measured to be 16 based on integration of the noise region adjacent to the analyte peak over the same time window as for the peak integration.



**Fig. 2.** GALC-P ion counts (integral of the multiple reaction monitoring ion chromatogram) divided by the GALC-IS ion counts as a function of the fraction of Pompe disease cell lysate (GM13763-HIGH) added to the Krabbe cell lysate (GM06805-LOW).

Total protein per assay was either 2 or 5  $\mu\text{g}$ . The right panel is an expansion of the plot in the left panel. Assays were performed in triplicate, and all assay values are plotted. The nonzero  $y$  intercepts are due to a non-GALC enzyme(s) in GM06805-LOW lysate capable of a small amount of conversion of GALC-S to GALC-P.

We explored a number of previously reported glycohydrolase inhibitors to see if the assay response in GM06805-LOW cells could be reduced toward the value seen in the no-lysate control. We also tested the inhibitor on GM13793-HIGH cells to explore the effect of the compound on GALC. The results, summarized in the online Supplemental Material file, suggested that the residual activity in GM06805-LOW cells was due to one or more enzymes other than GALC, possibly GBL1. The results also showed that the buffer composition had a large effect on the substrate specificity of enzymes in the cell lysates capable of cleaving GALC-S.

#### ANALYTICAL RANGE AND ACCURACY OF DETECTION OF SMALL AMOUNTS OF RESIDUAL GALC ACTIVITY

We defined the analytical range as the enzyme-dependent assay response observed with the HIGH quality control sample (GM13793-HIGH, typical of the activity in cells from a healthy individual) divided by the assay response from all enzyme-independent processes. The analytical range for the LC-MS/MS, discussed in detail in the online Supplemental Material file, had a value of 149. As shown in the online Supplemental Material file, the largest contribution to the enzyme-independent assay response was conversion of GALC-S to GALC-P by one or more non-GALC components of the cell lysate.

Since the analytical range for the LC-MS/MS GALC assay was larger than that for previously reported GALC assays (see below), we tested the possibility of

detecting a difference in assay response due to very small changes in the amount of GALC in cell lysates. To study this, we added small amounts of GALC-containing lysate (GM13793-HIGH) to GALC-null cell lysate (GM06805-LOW) so that the total amount of cellular protein was the same in all assays (2 or 5  $\mu\text{g}$ ). The results are shown in Fig. 2. Assays were conducted in triplicate, and the mean assay response was obtained along with the estimated error. The LC-MS/MS ion chromatograms are shown in online Supplemental Fig. 2. The data showed that very small differences in GALC enzymatic activity at the low end could be observed in a statistically significant way. For example, the ratio of GALC-P to GALC-IS ion counts when 100% GM06805-LOW lysate was used was 0.0012 (0.000044), and it was 0.00014 (0.000015) for the no-lysate blank. The difference in these values was 0.0011 (0.000046) and was thus of high confidence. This difference was 8-fold the value for the no-lysate control. The GALC-P/GALC-IS ion count ratio observed with 100% GM13793-HIGH lysate was 0.2999, thus the 100% GM06805-LOW lysate produced 0.4% of the GALC activity as the GM13793-HIGH lysate. Under our assay conditions, this was the contribution by one or more non-GALC enzymes in the lysate (possibly GBL1).

As shown in Fig. 2 (right), when 5  $\mu\text{g}$  of cell lysate protein was used per assay, an increase in GALC activity above that measured with 100% null lysate (GM06805-LOW) was readily measured when this lysate was spiked

**Table 2.** GALC activities measured by LC-MS/MS in T-lymphocytes isolated from patient blood.<sup>a</sup>

Patient	Approximate age of onset of symptoms	Age at time of blood draw	GALC activity (% of GM13763-HIGH), mean $\pm$ SD <sup>b</sup>	Psychosine in blood, nmol/L
EIKD-1	<1 month	<1 month	0.49 $\pm$ 0.09 (0.42 $\pm$ 0.08) <sup>b</sup>	15.7
EIKD-2	<1 month	<1 month	0.35 $\pm$ 0.02 (0.38 $\pm$ 0.04) <sup>b</sup>	26.1
EIKD-3	<1 month	<1 month	0.30 $\pm$ 0.08 (0.29 $\pm$ 0.08) <sup>b</sup>	20.4
LOKD-1 (late infantile)	10 years	12 years	1.72 $\pm$ 0.31 (1.79 $\pm$ 0.28) <sup>b</sup>	6.8
High Risk-KD-1	asymptomatic, high risk	<1 month	1.46 $\pm$ 0.17	3.0
Moderate Risk-KD-1	asymptomatic, moderate risk	<1 month	11.02 $\pm$ 1.28	0.4
Moderate Risk-KD-2	asymptomatic, moderate risk	<1 month	5.78 $\pm$ 0.24	0.5

<sup>a</sup> EIKD has onset of symptoms at <1 month. High Risk-KD and Moderate Risk-KD patients are asymptomatic newborns (see main text for description of risk category). Error bars are the SDs from triplicate analyses.

<sup>b</sup> Numbers in parentheses are repeat triplicate analyses done after 4 months of storage at  $-80^{\circ}\text{C}$  of the lysates assayed originally.

with 0.5% GM13793-HIGH lysate. The activity increased linearly as the fraction of GM13793 was increased.

We believe that it will be clinically useful to compare this discrimination between small differences in GALC activity to what was feasible with the previously developed assay of GALC, which was used to support follow-up studies of Krabbe disease newborn screening-positive patients (9). The previous GALC assay made use of radiolabeled galactosylceramide that contained tritium attached to C6 of the galactosyl group (6). Using the specific radioactivity of the tritiated galactosylceramide substrate, the micrograms of peripheral blood mononuclear cell (mainly lymphocytes) protein per assay, and the reported GALC activity (6), approximately 400 disintegrations per minute (dpm) of tritium were detected using this radiometric assay. We have carried out this radiometric assay and observed an assay response of 406 and 433 dpm for duplicate runs using 50  $\mu\text{g}$  of cell protein per assay (see online Supplemental Material) (6). This number agrees with the published value (6). In a control assay with omission of cell lysate, we observed assay responses of 49 and 53 dpm for duplicate runs (see online Supplemental Material). Thus, the analytical range for this assay was  $(420 - 50)/50 = 7.4$ , which was 20-fold less than that for the LC-MS/MS GALC assay. As noted above, an increment in GALC activity corresponding to 0.4% of that measured with GM13793-HIGH was observed with the LC-MS/MS with high confidence. This corresponded in the radiometric GALC assay to  $0.004 \times (420 - 50) = 1.5$  dpm, which was only 3% of the dpm value measured in the no-lysate control. When we carried out the no-lysate control as 5 replicates we obtained a mean (SD) blank value of 49 (3) dpm. Clearly it was not possible to measure increments of GALC activity with the radiometric assay of <2%–3% of normal activity.

Also of note, the radiometric assay was performed with 50  $\mu\text{g}$  of cell lysate protein and required 2–3 mL of patient blood, whereas the LC-MS/MS assay was performed with 10- to 20-fold less protein and 0.5 mL of patient blood was sufficient for >15 GALC assays, thus allowing triplicate assays to be routinely performed. It is difficult to obtain >2–3 mL of blood from newborns.

#### GALC ACTIVITY IN T LYMPHOCYTES FROM KRABBE DISEASE PATIENTS

We performed LC-MS/MS assays to measure the small amounts of residual GALC enzymatic activity in T lymphocytes prepared from venous blood drawn from 8 patients. Three patients [early-infantile Krabbe disease (EIKD)-1, -2, -3] were infants confirmed by clinical examination to have early infantile Krabbe disease, at which time the blood was drawn for LC-MS/MS GALC assay. One patient [late-onset Krabbe disease (LOKD), Table 2] was 12 years old at the time of the blood collection and became symptomatic for Krabbe disease in the late-infantile period (stage II symptoms (10)). Three infants were identified by the New York newborn screening program but did not have symptoms of Krabbe disease (High Risk-KD-1, Moderate Risk-KD-1, Moderate Risk-KD-2). The high-risk patient was placed in the high-risk category by the New York Krabbe disease consortium based on a radiometric GALC activity in the lowest bracket ( $\leq 0.15$  nmol product/h/mg protein), whereas moderate-risk patients were assigned to the moderate-risk category (next bracket up). Results are shown in Table 2. This table shows the repeat triplicate GALC assay results on the 3 EIKD patients and the 1 LOKD patient after the lymphocytes were stored for 4 months at  $-80^{\circ}\text{C}$ . The interassay variation was small in that the same difference was seen in the GALC values between the EIKD and LOKD patients as was seen earlier. The %CV for approx-

Table 3. Data for 14 patients identified by the New York Krabbe disease newborn screening program.					
Patient	Radiometric GALC activity, nmol/h/mg protein	Genotype		Krabbe disease status	
EIKD-4	0.01	p.R168C	g.30Kb Del // p.R168C	g.30 Kb Del	Infantile Krabbe disease
EIKD-5	0.05	p.R168C	g.30 Kb Del // p.I546T	p.X670Qext42	Infantile Krabbe disease
EIKD-6	0.02	p.R168C	g.30Kb Del // p.R168C	g.30 Kb Del	Infantile Krabbe disease
EIKD-7	0.12	p.R168C	g.30Kb Del // p.G360DfsX2		Infantile Krabbe disease
High Risk-KD-2	0.06				Asymptomatic
High Risk-KD-3	0.12				Asymptomatic
High Risk-KD-4	0.07				Asymptomatic
High Risk-KD-5	0.09				Asymptomatic
High Risk-KD-6	0.12				Asymptomatic
High Risk-KD-7	0.03				Asymptomatic
High Risk-KD-8	0.05				Asymptomatic
High Risk-KD-9	0.05				Asymptomatic
High Risk-KD-10	0.06				Asymptomatic
High Risk-KD-11	0.07				Asymptomatic

imately 20 repeats of our Quality Control High (GM13763-HIGH lymphoblasts) carried out over an approximately 1-year period was 6%.

All 3 early infantile Krabbe disease patients showed GALC activity in T lymphocytes that was the same as that seen in GM06805-LOW LCL lymphoblasts, which are null for GALC. On the other hand, the late-onset Krabbe and the asymptomatic newborn patients had statistically significant higher levels of GALC. The number of patients analyzed was too small to make a firm conclusion that the new GALC assay can better predict the onset of Krabbe disease.

Table 3 shows the radiometric GALC activity in lymphocytes for 14 patients previously identified by the New York Krabbe disease newborn screening program. All 14 patients were placed in the high-risk category by the newborn screening program based on leukocyte radiometric GALC of  $\leq 0.15$  nmol product/h/mg protein (Table 3). In this table genotypes are given along with Krabbe disease status. Four patients were confirmed by clinical examination to have early infantile Krabbe disease. The remaining 10 patients have not displayed Krabbe disease symptoms as far as we know. All 4 patients with early infantile Krabbe disease had 2 severe mutations in both copies of the *GALC* gene (either the 30-kb deletion or a frame shift or a C-terminal extension of 42 amino acids). There was no general trend that the radiometric GALC assay gave lower enzymatic activity for symptomatic vs asymptomatic patients. In fact, 1 of the symptomatic patients (EIKD-4) displayed a GALC activity of 0.12 nmol/h/mg protein, which was 12-fold higher than the activity measured in EIKD-1 and was as

high or higher than GALC activity in all of the asymptomatic patients. EIKD-4 is homozygous for the 30-kb deletion and should be devoid of functional GALC protein. Thus, the radiometric GALC assay gave activity values that were not correlated with strong predictions based on severe genotypes or with the severity of Krabbe disease. Estimated errors in the values in Table 3 were not available.

#### STABILITY OF GALC IN WHOLE BLOOD

Freshly drawn blood must be shipped to the biochemical genetics laboratory where the GALC assay will be performed. It was thus important to study the stability of GALC in whole blood before isolation of T lymphocytes. Whole blood was collected into a K2EDTA collection tube, and 0.5-mL aliquots were stored at ambient temperature (approximately 21 °C) or at 4 °C for up to 5 days. At regular intervals, T lymphocytes were isolated and submitted to LC-MS/MS assay of GALC enzymatic activity. Blood stored up to 3 days at 4 °C lost  $\leq 15\%$  of the initial GALC activity, whereas blood stored at ambient temperature for up to 3 days lost up to 30% of the initial GALC activity (Table 4). On the basis of these results, we recommend that blood be collected, stored at 4 °C until shipment the same day, and sent for arrival in  $< 3$  days with frozen gel packs at approximately 4 °C.

#### PSYCHOSINE ANALYSIS IN DRIED BLOOD SPOTS FROM KRABBE PATIENTS

We also measured the concentration of psychosine (galactosylsphingosine) in dried blood spots from the patients listed in Table 2. Psychosine may be a physiologi-

**Table 4** Whole blood GALC stability studies.

Blood storage	GALC activity, nmol/h/mg
Fresh blood (adult 1)	3.66
3 Days at 4 °C storage (adult 1)	3.91
Fresh blood (adult 2)	3.72
1 Day at ambient temperature (adult 2)	2.18
2 Days at ambient temperature (adult 2)	2.10
3 Days at ambient temperature (adult 2)	2.84
Fresh blood (adult 3)	3.55
1 Day at 4 °C (adult 3)	3.02
2 Days at 4 °C (adult 3)	3.15
3 Days at 4 °C (adult 3)	3.25

cally relevant substrate for GALC, and recent studies have shown that psychosine is increased in patients with early-infantile Krabbe disease but not in asymptomatic patients (11, 12). All 3 early infantile Krabbe disease patients had greatly increased psychosine concentration in blood (15.7, 20.4, and 26.1 nmol/L) compared to the other samples. Of note, among the 3 asymptomatic newborns, psychosine was increased 6- to 8-fold in the patient with the lowest GALC activity among the group (1.46% vs 5.78% and 11.2%). Moderate Risk-KD-1 and Moderate Risk-KD-2 patients with 11.02% and 5.78% of GALC activity, respectively, showed a low concentration of psychosine (0.4–0.5 nmol/L), values similar to those seen in 100 random newborns (0.3–0.5 nmol/L, data not shown). The patient with late-onset Krabbe disease also showed an intermediate increased psychosine of 6.8 nmol/L. These results suggest that the degree of psychosine increase correlates with the level of GALC enzymatic activity. The results also showed that an increase in GALC activity of just a few percent near the low led to a lack of psychosine accumulation to any measurement ex-

tent; thus, it was critical to be able to measure GALC enzymatic activity very accurately in the 0%–5% range. The LC-MS/MS method developed in this study showed the analytical resolution to accomplish this. Further studies may prove whether measurement of psychosine or accurate GALC activity or both is best at predicting the severity of Krabbe disease.

#### PROSPECTS FOR KRABBE DISEASE DIAGNOSIS AND PREDICTION

The main purpose of this report was to show that it was possible to develop an assay of GALC that had a much higher accuracy than the currently used radiometric GALC assay. The preliminary data on a small number of patients supports the new LC-MS/MS GALC as having the potential to predict which patients will develop Krabbe disease early in life, later on, or not at all.

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## References

- Wenger DA, Rafi MA. Krabbe disease: one hundred years from the bedside to the bench to the bedside. *J Neurosci Res* 2016;94:982-9.
- Escolar ML, West T, Dallavecchia A, Poe MD, LaPoint K. Clinical management of Krabbe disease. *J Neurosci Res* 2016;94:1118-25.
- Escolar ML, Poe MD, Provenzale MJ, Richards KC, Allison J, Wood S, et al. Transplantation of umbilical-cord blood in babies with infantile Krabbe's disease. *NEJM* 2005;352:2069-81.
- Orsini JJ, Kay DM, Saavedra-Matiz CA, Wenger DA, Duffner PK, Erbe RW, et al. Newborn screening for Krabbe disease in New York State: the first eight years' experience. *Genet Med* 2016;18:239-48.
- Gelb MH, Scott CR, Turecek T. Newborn screening for lysosomal storage diseases. *Clin Chem* 2015;61:335-46.
- Wenger DA, Sattler M, Clark C, McKelvey H. An improved method for the identification of patients and carriers of Krabbe's disease. *Clin Chim Acta* 1974;56:199-206.
- Luzi P, Rafi MA, Wenger DA. Characterization of the large deletion in the GALC gene found in patients with Krabbe disease. *Hum Mol Genet* 1995;4:2335-8.
- Li Y, Scott CR, Chamoles NA, Ghavami A, Pinto BM, Turecek F, Gelb MH. Direct multiplex assay of lysosomal enzymes in dried blood spots for newborn screening. *Clin Chem* 2004;50:1785-96.
- Duffner PK, Caggana M, Orsini JJ, Wenger DA, Patterson MC, Crosley CJ, et al. Newborn screening for Krabbe disease: the New York State model. *Pediatr Neurol* 2009;40:245-52.
- Escolar ML, Poe MD, Martin HR, Kurtzberg J. A staging system for infantile Krabbe disease to predict the outcome after unrelated umbilical cord blood transplantation. *Pediatr* 2006;118:e879-89.
- Matern D, Turgeon CC, Orsini JJ, Sanders KA, Hessemann J, Oglesbee D, et al. Measurement of psychosine in dried blood spots—a possible improvement to newborn screening programs for Krabbe disease. *J Inher Metab Dis* 2015;38:923-9.
- Chuang WL, Pacheco J, Zhang XK, Martin MM, Biski CK, Keutzer JM, et al. Determination of psychosine concentration in dried blood spots from newborns that were identified by newborn screening to be at risk for Krabbe disease. *Clin Chim Acta* 2013;419:73-6.