Establishing New Cut-Off Limits for Galactose 1-Phosphate-Uridyltransferase Deficiency for the Dutch Newborn Screening Programme

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Abstract Newborn screening for classical galactosemia in the Netherlands is performed by five laboratories and is based on the measurement of galactose 1-phosphate-uridyltransferase (GALT) activity and total galactose (TGAL) in heel prick blood spots. Unexpected problems with the GALT assay posed a challenge to switch to a new assay. The aim of this study was to make an analytical and clinical evaluation of GALT assays to replace the current assay and to establish new cut-off values (COVs).

First, the manual assay from PerkinElmer (NG-1100) and the GSP assay were compared by analyzing 626 anonymous heel prick samples in parallel. Secondly, a manual GSP method was evaluated and 2,052 samples were compared with the

automated GSP assay. Finally, a clinical evaluation was performed by collecting data from 93 referred newborns.

No satisfactory correlation was observed between GALT activity measured with the manual NG-1100 assay and the automated GSP assay. An acceptable correlation was found between the manual and automated GSP assay. Intra- and inter-assay variation of the automated GSP were 1.8–10.0% and 3.1–13.9%, respectively. Evaluation of clinical data demonstrated that adjusting the COVs for GALT to 2.0 U/dl and TGAL to 1,100 $\mu\text{mol/l}$ improved specificity of screening for classical galactosemia.

An assay designed for automated processing to measure GALT activity in heel prick samples works equally well when processed manually. We therefore adopted both methods in the Dutch screening laboratories. As a result of this evaluation new COVs for GALT and TGAL have been introduced and are valid from July 2015.

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Introduction

Classical galactosemia (OMIM 230400) is an autosomal recessively inherited disorder of galactose metabolism caused by a severe deficiency of galactose 1-phosphate-uridyltransferase (GALT) enzyme (GALT; EC 2.7.7.12), (Isselbacher et al. 1956). Its incidence in Western Europe has been estimated to be between 1:23,000 and 1:44,000 (Bosch 2006). Affected children develop a severe illness with feeding difficulties, liver failure, *Escherichia coli* sepsis, and cataracts after ingestion of galactose from breast milk or infant formula in the first weeks of life. Timely treatment, consisting of a strict restriction of dietary galactose, is lifesaving, rendering this disease well suited for newborn screening.

The Dutch newborn screening for classical galactosemia, which was implemented in 2007, is primarily based on the measurement of GALT activity in filter paper blood spots. In heel prick samples with an activity level at or below 20% of average GALT activities of daily experimental batches, total galactose (TGAL) concentration was determined in the same blood sample (cut-off value (COV) for positive screening result and clinical referral was 700 $\mu\text{mol/l}$ blood). The five Dutch screening laboratories aim to work with the same laboratory equipment and assays if possible to use the same, nationwide, COVs. For the galactosemia screening, three laboratories have experience using an automated GALT assay (PerkinElmer, 3303-0010-assay using the GSP analyzer) while two laboratories use a manual (Bio-Rad) assay.

Based on the data from the yearly reports of the newborn screening laboratories, in 2010–2014 (not publically available) about 0.4% of the newborn heel prick samples were considered abnormal and children were referred to a metabolic center; roughly 5% of referred children were diagnosed with classical galactosemia and roughly 6% were Duarte variants demonstrating GALT activity of 15–30%. So far, no cases of uridine diphosphate galactose 4-epimerase deficiency were found in the Netherlands.

Rather sudden delivery problems with the manual GALT assay in 2013 posed an immediate challenge for two laboratories to switch to a new assay that would fit as seamlessly as possible to the logistics and COVs of the current screening programme.

The aim of this study was to evaluate new GALT assays in order to fit these assays in the Dutch screening programme, maintaining uniformly applied COVs for GALT. Here we report the analytical and clinical evaluation of the new GALT assay in the context of the Dutch screening setting. The consequences for the Dutch newborn screening programme, including the COV for galactose, are discussed.

Methods

Study Population and Samples

Samples were selected from the routine screening programme in the laboratories immediately after performing the routine newborn screening. Approval to use anonymized samples for quality assurance properties and to improve test quality was obtained from the parents through the heel prick application form (in case parents notified informed dissent, cards were not used).

Assays

Three laboratories already had experience using the automated GALT assay (3303-0010-assay, PerkinElmer, Turku, Finland) and using the Genetic Screening Processor (GSP analyzer, PerkinElmer). The first choice for evaluation by the remaining two other laboratories was the manual GALT assay of PerkinElmer (NG-1100). An alternative method was to test whether the GSP assay could be used without a GSP (further referred to as manual GSP assay). To this end, the incubation steps carried out by the GSP analyzer were performed manually and the plates were read using a Fluorometer (Victor, PerkinElmer). This idea was originally suggested by Fingerhut and Torresani (2013).

Analytical Procedures

The experimental procedures for the manual and automated assays are comparable and were performed according to the manufacturer's protocol. Using a DBS puncher (Perkin Elmer), single 3.2 mm punches were collected in 96 well plates and incubated in a substrate reagent. The method applied in both GALT assays is an adaptation of the semiquantitative enzymatic assay of Beutler and Baluda (1966). GALT activity is determined using a coupled enzyme reaction, in which the end product (fluorescent NADPH) is measured by excitation with a laser source at 355 nm and detecting emitted light at 460 nm. Measurement of fluorescence was done using a Victor Fluorometer (PerkinElmer, 1420 Multilabel Counter) or with the GSP. Using this test, patients with GALT deficiency are identified, but also deficiencies of glucose-6-phosphate dehydrogenase and phosphoglucomutase (Tegtmeyer et al. 2014) can be picked up, since these enzymes are required for the test to function. The NG-1100 Newborn GALT kit for manual processing is provided with calibrators to express GALT activity in units per gram hemoglobin. The 3303-0010 GSP Newborn GALT kit was processed with the GSP automated analyzer and is provided with calibrators to express GALT activity in units per dl blood. Thus, both methods differ compared to the method routinely used, where GALT was expressed as percentage of the average of the daily batch.

For the comparative assays, experimental runs of 100–200 samples were analyzed in the routine daily screening in the laboratory of Bilthoven using the automated GSP method. Duplicate punches were taken to fill duplicate plates that were transported for parallel analysis at the laboratory of the Academic Medical Center in Amsterdam, where the NG-1100 assay was performed the next

day. For the comparison between the automated GSP and NG-1100 assay a total of 626 daily routine samples was used. For the comparison of both GSP assays (automated GSP versus manual GSP) a total of 2,052 daily routine samples and a selected group of 68 samples with low GALT activities were measured. In addition, intra- and inter-assay variation was determined for the automated and manual GSP assay.

Finally, data were collected from 93 newborns with low GALT activities (performed by the automated and manual GSP method) who were referred to the hospital for further analysis. Based on these data optimal COVs for GALT were assessed.

Statistical Analysis

Comparison was established using Passing–Bablok statistics and Pearson correlation (Analyse-it software, which is integrated in Microsoft Excel 2010). Intra- and inter-assay variation (as % coefficient of variance (%CV) where %CV is the quotient of SD and average $\times 100$) were also established.

Results

Firstly, the manual assay (NG-1100) and the automated GSP assay were compared by analyzing 626 anonymous routine heel prick samples in parallel. Figure 1 shows the GALT activity measured by both assays. We did not observe a satisfactory correlation between both assays (Pearson correlation: $r = 0.37$ (95% confidence interval:

0.30–0.43) and Passing–Bablok analysis: slope = 0.37 and an intercept of 3.24).

Secondly, the manual GSP method was carried out. Comparative results of 2,052 measurements using the 3303-0010-assay, performed either automated or manually, are given in Fig. 2a. With a slope of 0.86, an intercept of -0.8 and a correlation coefficient of 0.86 (0.85–0.87) between the manual and automated GSP method were deemed sufficiently comparable. In addition, data of a selected group of heel prick samples with low GALT values (<6.0 U/dl) were measured using both assays (Fig. 2b). Here, it is shown that the results were comparable to those shown in Fig. 2a, although GALT results of the manual assay were slightly lower compared to the automated GSP assay (with a slope of 0.74, intercept of 0.31, and correlation 0.64 (0.47–0.76)).

Intra- and inter-assay variation coefficients of the automated and manual GSP assay are listed in Table 1. Both assays showed acceptable variation coefficients (ranging from 1.8 to 13.9%) where the automated GSP assay performed better than the manual GSP assay.

Figure 3 reports the data collected from all newborns that were referred by all Dutch screening laboratories between July 2014 and June 2015 based on provisionally applied COVs (GALT activity ≤ 2.7 U/dl and TGAL ≥ 900 $\mu\text{mol/l}$). Two newborns were excluded from this evaluation, since referral data were incomplete. Ninety-three newborns were referred (0.05% of all newborns screened in this period). In this group, three patients were confirmed to have classical galactosemia, and 41 newborns had GALT activities that are indicative of a Duarte variant. All patients with classical galactosemia showed GALT activities ≤ 0.8 U/dl combined with TGAL concentrations $\geq 3,000$ $\mu\text{mol/l}$. The positive predictive value (PPV) was 3.3% (Duarte variants being considered as false positive results).

Discussion

The present study describes the adoption of a manual assay for GALT used in the newborn screening for classical galactosemia in order to replace the assay of which the production was terminated. Choices were limited, and of those limited choices we showed that the manual GALT assay from Perkin Elmer (NG-1100 assay) did not produce results that were comparable with those measured using the automated GALT assay (3303-0010-assay, processed with the GSP analyzer). In additional experiments we demonstrated that adaptation of the automated GSP assay for manual processing did produce satisfactory results. Intra- and inter-assay variation were acceptable and comparable in both assays and, more importantly, a good correlation between the measurements of the automated and manual

Fig. 1 GALT activity measured in 626 daily heel prick samples comparing the manual assay with the automated GSP assay

Fig. 2 GALT activity measured in 2,052 random heel prick samples (a) and in a selection of 68 heel prick samples (b) with GALT activity ranging from 0 to 6 U/dl using the automated assay and manual GSP

assay. Slope and intercept of the Passing–Bablok analysis (*grey line*) are given in the diagram

Table 1 Intra- and inter-assay variation of GALT activity measured by the manual and automated GSP assay of low and medium control samples

	Low control (3.7 U/dl)		Medium control (13.8 U/dl)	
	Automated GSP	Manual GSP	Automated GSP	Manual GSP
Intra-assay				
Average	3.7	3.7	14.5	12.5
CV (%)	7.0	10.0	1.8	4.2
Inter-assay				
Average	3.7	3.8	13.8	12.5
CV (%)	3.1	13.9	5.0	3.4

Intra-assay variation determined in one experiment with $n = 6$ analyses, inter-assay variation in ten experiments with 4–6 analyses

Fig. 3 Scattergram of results of all 93 newborns that were referred in a one year period (July 2014–June 2015) by all Dutch screenings laboratories based on GALT activity ≤ 2.7 U/dl and TGAL concentration ≥ 900 $\mu\text{mol/l}$ (gray line). The black line indicates new adjusted COVs (see the section “Discussion”). Referred neonates that appeared normal after clinical evaluation are indicated by open

diamonds, cases with the Duarte variant are indicated with gray circles, and classical galactosemia cases with black triangles. Note: analytical sensitivity for GALT was determined at 0.8 U/dl (based on our own validation studies), therefore results of ≤ 0.8 U/dl were all reported as 0.8 U/dl

GSP assay was shown. These results enabled implementation of this method in two of the five screening laboratories for measuring GALT activity in heel prick blood and set one nationwide-defined COV. The manual procedure was implemented on July 1, 2014. Concomitantly, a new COV for GALT, expressed in U/dl blood, had to be implemented.

The approach was to start with a relatively save, provisional COV, followed by a critical evaluation period, to allow fine tuning of the COV. In close collaboration with both the programme coordinator of the Dutch screening programme and the Dutch advisory board for newborn screening for metabolic disorders as of July 1, 2014, the initial COV for GALT activity in the Netherlands was set at GALT ≤ 2.7 U/dl blood (combined with TGAL ≥ 900 $\mu\text{mol/l}$). In a recent paper on GALT screening in the USA an overview was given of the applied COV for GALT screening (Pyhtila et al. 2014). Four out of nineteen laboratories used the PerkinElmer GSP assay, with COVs of 2.5, 2.7, 3.1, and 4.3 U/dl blood, respectively.

Thus, 2.7 U/dl as a COV for the Dutch screening programme, compared to all comparative reference data together, seemed to be a reasonable choice.

After one year of experience, we have no indication of false negative results of the screening using the current COVs. GALT activities in cases of classical galactosemia were ≤ 0.8 U/dl (Fig. 3; three patients) and in case of the possible Duarte variants 0.8–2.7 U/dl blood. TGAL concentrations of the classical galactosemia cases were $\geq 3,000$ $\mu\text{mol/l}$ and of the Duarte variants between 900 and 2,717 $\mu\text{mol/l}$. Duarte variants are considered to be physiological variants and do not receive treatment.

To optimize COVs for GALT after the planned evaluation period we combined the limited available clinical and screening data of the current study and combined clinical and screening data (produced by using the exact same automated GSP method as the current study) from the Swiss newborn screening programme (kindly provided by

Dr. Fingerhut). In the Swiss study it was shown that GALT activity of five classical galactosemia cases was 0–1.4 U/dl and of 10 Duarte cases GALT varied between 0.7 and 3.3 U/dl. TGAL concentrations of the classical galactosemia cases ranged from 965 to 7,148 $\mu\text{mol/l}$ blood and of the Duarte casus from 19 to 2,368 $\mu\text{mol/l}$ blood. GALT activity results together led us to believe that a new COV for GALT activity to detect neonates with classical galactosemia only could safely be set at 2.0 U/dl.

To set a new COV for TGAL we sought additional information on the lowest TGAL concentration (965 $\mu\text{mol/l}$) in this dataset measured in a patient with classical galactosemia. It turned out that this patient was already on a galactose-free diet, because of the presence of a sibling with classical galactosemia.

It was therefore decided to increase the TGAL COV from 900 to 1,100 $\mu\text{mol/l}$. These new COVs (GALT $\leq$ 2.0 U/dl and TGAL $\geq$ 1,100 $\mu\text{mol/l}$) were approved by the National Screening Programme Committee and are valid as of July 1, 2015. Adjustment of the COVs will result in a significant decrease of the referral rate (48 instead of 93, projected on the July 2014–June 2015 data) and thereby reducing the amount of false positive referrals (45 instead of 90) and a subsequent increase in the PPV (6.5% instead of 3.3%). Duarte variants are included in the number of false positives. Adjustment of the COVs results in increased specificity (99.98%) compared to the formerly used COVs (specificity 99.95%) with unchanged sensitivity (100%).

The finding of one neonate with classical galactosemia that presented in 2011 with a very low GALT activity but a TGAL concentration of 959 hampered the decision on a COV of 1,100 $\mu\text{mol/l}$ blood for some time. The fact that this particular neonate at the time of the heel prick was already on a partly galactose-free diet not only paved the way to elevate the COV for TGAL, but also stressed the importance of the dietary status of the neonate with respect to galactosemia screening. A question on the Dutch heel prick card might be a future consideration, allowing for an even higher TGAL COV, and thus a lower referral rate.

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Take-Home Message

A new method for measuring GALT activity for newborn screening was introduced in the Netherlands and has led to

new COVs in newborn screening for classical galactosemia and may lower referral rates.

Contributions of Individual Authors

Boelen A: Conception and design, data analysis and interpretation of data, drafting and revising article.

Kemper EA: Conception and design, data analysis and interpretation of data, drafting and revising article.

Bosch AM: Interpretation of data, professional consultation, revising article.

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Compliance with Ethics Guidelines

Conflict of Interest

Anita Boelen, Evelien Kemper, Annet Bosch, Marja van Veen, Carolien van Rijswijk, Marelle Bouva, Ralph Fingerhut, and Peter Schielen declare that they have no conflict of interest. With regard to the mandatory submission of the Conflict of Interest Disclosure Form, please see the following section on “Competing Interests”.

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