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The Galactose Index measured in fibroblasts of GALT deficient patients distinguishes variant patients detected by newborn screening from patients with classical phenotypes

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ABSTRACT

Background: The high variability in clinical outcome of patients with Classical Galactosemia (CG) is poorly understood and underlines the importance of prognostic biomarkers, which are currently lacking. The aim of this study was to investigate if residual galactose metabolism capacity is associated with clinical and biochemical outcomes in CG patients with varying geno- and phenotypes.

Methods: Galactose Metabolite Profiling (GMP) was used to determine residual galactose metabolism in fibroblasts of CG patients. The association between the galactose index (GI) defined as the ratio of the measured metabolites [$U^{13}C$]Gal-1-P/ [$^{13}C_6$]UDP-galactose, and both intellectual and neurological outcome and galactose-1-phosphate (Gal-1-P) levels was investigated.

Results: GMP was performed in fibroblasts of 28 patients and 3 control subjects. The GI of the classical phenotype patients (n = 22) was significantly higher than the GI of four variant patients detected by newborn screening (NBS) (p = .002), two homozygous p.Ser135Leu patients (p = .022) and three controls (p = .006). In the classical phenotype patients, 13/18 (72%) had a poor intellectual outcome (IQ < 85) and 6/12 (50%) had a movement disorder. All the NBS detected variant patients (n = 4) had a normal intellectual outcome (IQ ≥ 85) and none of them has a movement disorder. In the classical phenotype patients, there was no significant difference in GI between patients with a poor and normal clinical outcome. The NBS detected variant patients had significantly lower GI levels and thus higher residual galactose metabolism than patients with classical phenotypes. There was a clear correlation between Gal-1-P levels in erythrocytes and the GI (p = .001).

Conclusions: The GI was able to distinguish CG patients with varying geno- and phenotypes and correlated with

Abbreviations: CG, classical galactosemia; GALT, galactose-1-phosphate uridylyltransferase; Gal-1-P, galactose-1-phosphate; NBS, newborn screening; GI, galactose index; GMP, Galactose metabolite profiling; IQ, intelligence quotient; MD, movement disorder; GC–MS, gas chromatography mass spectrometry; CV, coefficient of variation

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Gal-1-P. The data of the NBS detected variant patients demonstrated that a higher residual galactose metabolism may result in a more favourable clinical outcome. Further research is needed to enable individual prognostication and treatment in all CG patients.

1. Introduction

Classical Galactosemia (CG) is an autosomal recessive inborn error of galactose metabolism. Due to a deficiency of the galactose-1-phosphate uridylyltransferase enzyme (EC 2.7.7.12; GALT) patients are unable to metabolize galactose, which leads to the accumulation of galactose-1-phosphate (Gal-1-P) and galactitol. The only available treatment is a galactose restricted diet. An early onset of the diet (after newborn screening (NBS) or family screening) improves neonatal outcome, but an early onset of and good compliance with the diet do not prevent long-term complications such as cognitive impairment, movement disorders and in females primary ovarian insufficiency [1–5]. The pathophysiology of CG and the broad clinical outcome spectrum ranging from fully normal to severely impaired are poorly understood. In patients with a GALT deficiency, Gal-1-P is persistently elevated even in dietary adherent patients, due to the endogenous production of galactose [6,7]. Both the accumulation of Gal-1-P and the reduced production of important substrates such as UDP sugars, are thought to contribute to the glycosylation defects demonstrated in CG patients and may thus contribute to the long-term complications [8–12]. At this time, there are no biomarkers that can predict the clinical outcome of CG patients. Prognostic biomarkers are urgently needed in all patients, but especially in patients with a classical phenotype in which clinical outcome varies highly and in patients detected since the implementation of NBS with previously unreported clinical and biochemical genotypes and phenotypes [13].

We hypothesize that differences in clinical outcome are caused by differences in residual galactose metabolism capacity. A slightly higher ability to metabolize galactose would cause lower Gal-1-P levels resulting in less abnormal galactosylation and possibly a more favourable clinical outcome. The method that is currently used in our cohort to measure erythrocyte GALT enzyme activity is not able to reliably detect differences in enzyme activity below 3.3% (< 1.1 umol/h.g Hb). To study the correlation between residual enzyme activity and clinical outcome, other methods should be investigated that are able to reliably detect (even slight) differences in residual galactose metabolism capacity. Metabolite profiling in fibroblasts has been demonstrated to correlate well with clinical severity in other metabolic disorders such as inborn errors of fatty acid oxidation [14,15]. In our pilot study we found that galactose metabolite profiling (GMP) in cultured fibroblasts was a sensitive method to determine residual galactose metabolism capacity expressed as the Galactose Index (GI). This method was able to differentiate between patients with a classical phenotype, patients with a variant phenotype and controls [16].

The aim of this study was to determine if residual galactose metabolism measured in fibroblasts can be used as predictor of clinical outcome by investigating the association between the GI and both biochemical and clinical outcomes in CG patients with varying genotypes and phenotypes.

2. Materials and methods

2.1. Patients and controls

Galactose metabolite profiling (GMP) was performed in fibroblasts of GALT deficient patients with two known pathogenic variations in the *GALT* gene and/or an erythrocyte GALT enzyme activity < 15% of the reference mean and were collected for clinical or research purposes.

Table 1
Patient demographics and clinical outcomes.

Pt ID	Pt ID*	Group	*GALT*_1 / *GALT*_2	GALT activity, %	GI	IQ	MD
1	12	C	p.Gln188Arg / p.Gln188Arg	< 3.3	14.27	81	Yes
2	14	C	p.Gln188Arg / p.Gln188Arg	< 3.3	18.53	78	–
3a	13	C	p.Gln188Arg / p.Gln188Arg	< 3.3	16.65	77	Yes
4a	8	C	p.Gln188Arg / p.Gln188Arg	< 3.3	15.58	71	–
5	10	C	p.Gln188Arg / p.Gln188Arg	< 3.3	22.37	83	–
6	4	C	p.Gln188Arg / p.Gln188Arg	–	13.94	91	–
7	9	C	p.Gln188Arg / p.Gln188Arg	–	11.49	53	No
8	7	C	p.Gln188Arg / p.Gln188Arg	< 3.3	17.49	82	No
9	–	C	p.Gln188Arg / p.Gln188Arg	< 3.3	14.00	–	–
10	–	C	p.Gln188Arg / p.Gln188Arg	< 3.3	13.16	–	–
11	–	C	p.Gln188Arg / p.Gln188Arg	< 3.3	10.12	–	No
12	–	C	p.Gln188Arg / p.Gln188Arg	< 3.3	10.77	–	No
13	31	C	p.Gln188Arg / p.Lys127E	< 3.3	18.98	70	–
14	21	C	p.Gln188Arg / p.Ser135Trp	< 3.3	13.49	98	No
15	27	C	p.Gln188Arg / p.Lys285Asn	< 3.3	16.18	77	–
16	34	C	p.Ser135Trp / p.Arg51Gln	< 3.3	12.87	78	–
17b	17	C	p.Gln188Arg / p.Leu195Pro	< 3.3	9.04	52	Yes
18b	19	C	p.Gln188Arg / p.Leu195Pro	< 3.3	11.08	88	Yes
19	30	C	p.Gln188Arg / p.Lys127E	< 3.3	13.76	61	–
20	18	C	p.Gln188Arg / p.Leu195Pro	< 3.3	16.71	93	No
21c	26	C	p.Gln188Arg / p.Lys285Asn	< 3.3	13.73	86	Yes
22c	25	C	p.Gln188Arg / p.Lys285Asn	< 3.3	16.54	76	Yes
23	36	S	p.Ser135Leu / p.Ser135Leu	3.9	5.40	61	No
24	37	S	p.Ser135Leu / p.Ser135Leu	< 3.3	2.47	71	No
25	38	V	p.Gln188Arg / p.Met219Lys	7.2	4.52	96	No
26	39	V	p.Gln188Arg / c.1-96 T > G	3.6	6.31	86	No
27	–	V	p.Val128Ile / p.Val128Ile	9.3	7.17	91	No
28	–	V	p.Arg201His / p.Arg201His	8.9	1.61	–	–

* Patient (Pt) ID whole body galactose oxidation study. C: Classical phenotype, S: homozygous p.Ser135Leu, V: Variant patients, IQ: Intelligence Quotient, MD: Movement Disorder. a,b,c: sibs. -: missing data.

Table 2
Galactose Index (GI).

	N	Galactose Index
All patients	28	13.61 (1.61–22.37)
Classical phenotypes	22	13.97 (9.04–22.37)
NBS variant patients	4	5.42 (1.61–7.17)
Homozygous p.Ser135Leu	2	3.94 (2.47–5.40)
Controls	3	1.03 (0.84–1.12)

Notes. Data reported in median (ranges), NBS: newborn screening.

The fibroblasts collected for clinical reasons were used after patients or their parents gave informed consent. The research fibroblasts were collected from competent, adult patients after informed consent was obtained. All patients consented to the use of their clinical data for research purposes. This study was approved by the local medical ethics committee. The fibroblasts of healthy controls were collected for research purposes after informed consent and approval of the local medical ethics committee.

2.2. Patient groups

This cohort comprises GALT deficient patients with varying geno- and phenotypes; patients with classical phenotypes (two pathogenic *GALT* mutations and absent or barely detectable erythrocyte GALT activity), NBS detected variant patients (since 2007, with previously unreported geno- and phenotypes and erythrocyte GALT activity up to 10%, no clinical symptoms at diagnosis and undetectable Gal-1-P levels on dietary treatment) [13] and patients with the homozygous p.Ser135Leu genotype with residual GALT activity in other tissues than erythrocytes [17]. Patient demographics are listed in Table 1.

2.3. GMP measurements

The cell culture procedure and stable isotope ^{13}C labeled GMP measurements in fibroblasts were carried out as previously described [16]. In brief, fibroblasts were starved for 16 h in Dulbecco's phosphate buffered saline, followed by addition of 1 mM of [U^{13}C]Galactose. After an incubation period of 4 h metabolism was quenched and cells were processed for measurement of [U^{13}C]Galactose, [U^{13}C]Gal-1-P and [$^{13}C_6$]UDP-galactose. The galactose index (GI) was defined as the ratio of [U^{13}C]Gal-1-P/ [$^{13}C_6$]UDP-galactose. The GMP measurements were performed in triplicate and each patient cell line was measured in two independent experiments. Every experiment included at least two cell lines from control subjects and at least one cell line of a patient with a classical phenotype. The result (the GI) is the mean of the two independent experiments.

2.4. Clinical outcome

The clinical outcome parameters were intellectual and neurological outcome. In patients who received an age specific intelligence test, the IQ was used as derivative of intelligence. A poor intellectual outcome was defined as an IQ < 85 and a normal intellectual outcome as an IQ ≥ 85. The presence or absence of movement disorders (MDs) was used as a determinant for neurological outcome. Information was retrieved from the medical records of the included patients.

2.5. Biochemical outcome

The most recent Gal-1-P level documented in the medical charts of patients was used in this study. All included Gal-1-P levels were measured by gas chromatography mass spectrometry (GC–MS) in erythrocytes and were below 0.82 μmol/g Hb in diet adherent patients. Patients with self-reported dietary incompliance at the most recent Gal-

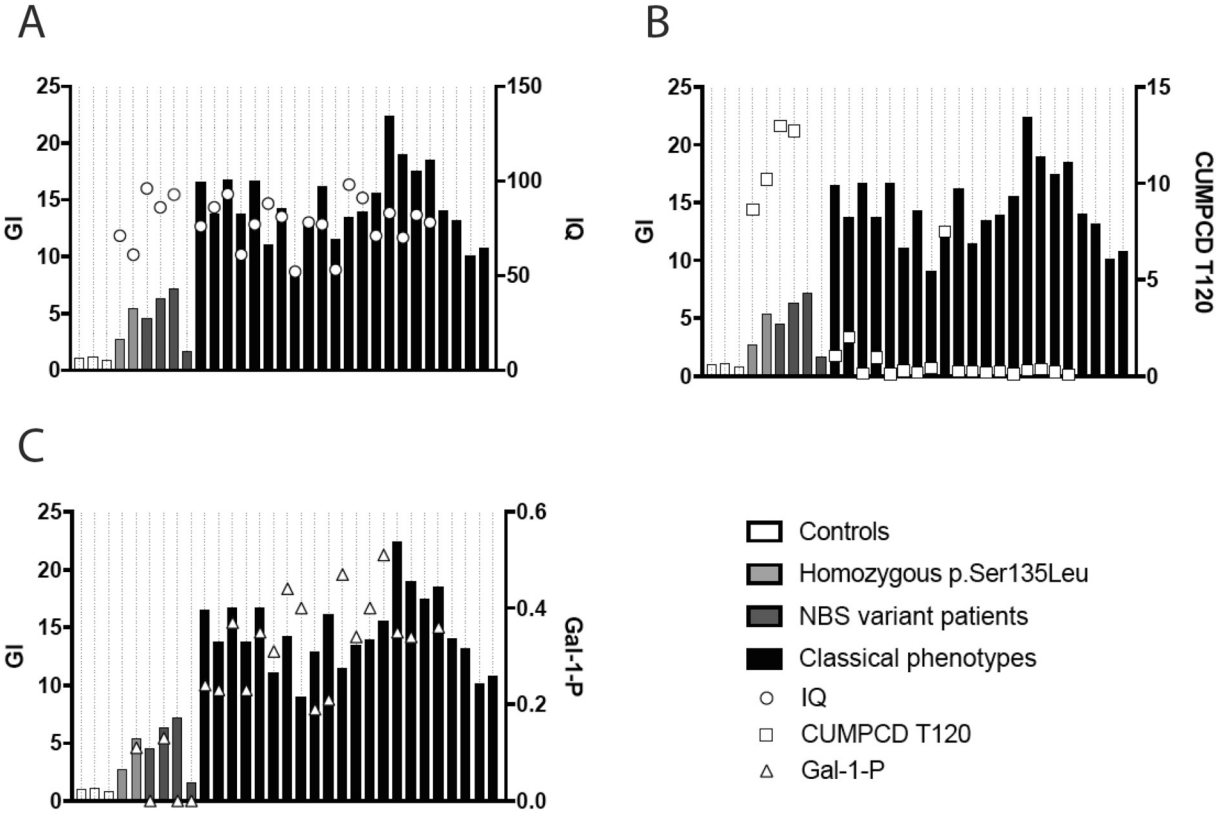

Fig. 1. Individual data on the Galactose Index (GI) and IQ (1A), GI and whole body galactose oxidation capacity after 120 min (CUMPCDT120) (1B) and GI and galactose-1-phophate (Gal-1-P) (1C) in Classical Galactosemia patients with varying phenotypes and controls.

1-P measurement were excluded from the Gal-1-P analysis.

2.6. Statistical analyses

SPSS version 25 (SPSS Inc. Chicago, Illinois, USA) was used to perform all statistical analyses. Data were presented as median and ranges because of a non-normal distribution. The Mann-Whitney *U* test was used to determine if statistically significant differences in GI were present between patients with a poor and normal clinical outcome. The Spearman's rank coefficient test was used to test for associations and regression analysis was used to test for correlations. *P*-values below 0.05 were considered statistically significant.

3. Results

3.1. Patients

Fibroblast samples of 28 patients were included in this study. Of the included patients, 22 were classified as patients with a classical phenotype (12 patients with the homozygous p.Gln188Arg genotype) and four as NBS variant phenotype. Two patients were homozygous for the p.Ser135Leu mutation. Residual GALT enzyme activity in erythrocytes was below the limit of quantitation of the enzyme assay (< 3.3%; < 1.1 μmol/h.g Hb) in 21/26 (Table 1). In two patients, the erythrocyte GALT enzyme activity was not documented.

3.2. GMP measurements

Galactose metabolite profiling (GMP) was performed in fibroblasts of 28 GALT deficient patients and three control subjects and the GI was calculated (Table 1, Table 2 and Fig. 1). The GI of the classical phenotype patients was significantly higher than of the NBS detected variants ($p = .002$), the homozygous p.Ser135Leu patients ($p = .022$) and controls ($p = .006$). The GI of the NBS detected variants was also significantly higher than of the controls ($p = .034$). The GI of the homozygous p.Ser135Leu patients was increased compared to the controls but the difference was not significant, probably due to the limited numbers ($p = .083$). The GI was significantly lower in pediatric patients when compared to adult patients ($p = .030$). In the classical phenotype group, there was no significant difference in GI between pediatric and adult patients. There was no significant difference in GI between males and females.

The results of the individual GMP measurements including the coefficient of variation (CV) are listed in the supplementary Table 1. One experiment contained several outliers and therefore the entire experiment (the GMP measurements of 7 classical phenotype patients, 2 variants and 2 controls in triplicate) was excluded.

3.3. The association between GI and clinical outcome

Our cohort included four NBS detected variant patients with previously unreported genotypes and higher residual GALT enzyme activity than the patients with classical phenotypes, and two homozygous p.Ser135Leu patients who were diagnosed at a later age (7 months and 10 years respectively) with residual GALT enzyme activity in other tissues. Since both the genotype and the late initiation of dietary treatment may influence clinical outcome, analyses were carried out both with and without these patients.

3.4. Intellectual outcome

The intellectual outcome of 24 patients was known, with an IQ ranging from 52 to 98 (median 78). The intellectual outcome was poor in 15/24 (IQ < 85) and normal in 9/24 (IQ ≥ 85). Overall, the GI in patients with a poor intellectual outcome was higher when compared to patients with a normal intellectual outcome, but the difference was not significant ($p = .053$) (Table 3). After the homozygous p.Ser135Leu patients were excluded, the GI became significantly higher GI in patients with a poor intellectual outcome ($p = .010$). In the group of classical phenotype patients, there was no significant difference in GI between patients with a poor and normal intellectual outcome. Overall, there was no correlation between the IQ (as continuous variable) and GI (Fig. 1A).

3.5. Neurological outcome

In our cohort, a movement disorder (MD; tremor and/or dystonia) was reported in 6/17 patients. Overall, the GI in patients with an MD was higher when compared to patients without an MD, but the difference was not significant ($p = .132$) (Table 3). The exclusion of the homozygous p.Ser135Leu patients did not change this finding. In the group of classical phenotype patients, there was no significant difference in GI between patients with and without an MD.

3.6. The association between GI and whole body galactose oxidation

This cohort includes 22 patients who have participated in a previous study, which investigated whole body galactose oxidation with the use of the 1-^{13}C galactose breath test [18].

Linear regression indicated a negative correlation between the GI and whole body galactose oxidation (F(1,20) 30.47, β-0.89 (95%CI -1.22– −0,55), $p < .0005$) (Fig. 1B). In the classical phenotype patients, there was no significant correlation between the GI and whole body galactose oxidation.

3.7. The association between the GI and biochemical outcome

For 21/28 patients, the most recent Gal-1-*P* value measured by GC–MS was retrieved from the medical charts. Three NBS detected variant patients demonstrated undetectable Gal-1-P levels (< 0.05 μmol/g Hb) under dietary treatment. Linear regression indicated a positive correlation between the GI and Gal-1-P (F(1,19) 13.89, β 2.19 (95%CI 0.96–3.43), $p = .001$) (Fig. 1C). In the group of classical phenotype patients, there was no significant correlation between Gal-1-P level and GI.

Table 3
Galactose index and clinical outcome.

	All patients		Classical phenotypes		NBS Variant patients		Homozygous p.Ser135Leu	
GI	N 28	13.49 (1.61–22.37)	N 22	13.97 (9.04–22.37)	N 4	5.42 (1.61–7.17)	N 2	\3.94 (2.47–5.40)
- IQ ≥ 85:	9	11.08 (1.61–16.71)	5	13.73 (11.08–16.71)	4	5.42 (1.61–7.17)	-	
- IQ < 85:	15	15.58 (2.47–22.37)	13	16.18 (9.04–22.37)	-		2	3.94 (2.47–5.40)
- MDs, No:	11	10.12 (2.47–17.49)	6	12.49 (10.12–17.49)	4	5.42 (1.61–7.17)	2	3.94 (2.47–5.40)
- MDs, Yes:	6	14.00 (9.04–16.65)	6	14.00 (9.04–16.65)	-		-	

Notes. Data reported in median (ranges), GI: Galactose Index, IQ: Intelligence Quotient, MD: Movement Disorder.

4. Discussion

In this study, we investigated the association between the GI measured in fibroblasts and the biochemical and clinical outcomes of GALT deficient patients. The previously developed GMP assay was used to determine residual galactose metabolism expressed as GI in a relatively large cohort of GALT deficient patients with varying geno- and phenotypes.

The results of this study demonstrate that the GI is able to differentiate between patients with a classical phenotype, NBS detected variant patients with possibly better outcomes and controls, and results are in line with the conducted pilot study [16]. Furthermore, GMP analysis is able to distinguish homozygous p.Ser135Leu patients from classical phenotype patients and controls.

An important issue in CG is the lack of predictors of clinical outcome, especially in patients with a classical phenotype who have highly variable outcomes. In our cohort of classical phenotype patients, the differences in GI between patients with a poor and normal clinical outcome were not significant and thus the GI was not able to predict clinical outcome. As the GI demonstrated considerable interpatient and inter-assay variation within the classical phenotype patients with severely deficient GALT enzyme activity, the question remains whether future studies in a larger cohort would be able to demonstrate significant differences in GI between classical patients with a poor and normal clinical outcome.

The variant patients detected by NBS have residual GALT enzyme activities up to 10% in erythrocytes, significantly lower Gal-1-P levels than classical patients and currently none of them demonstrates long-term complications. The GI of these patients is significantly lower when compared to classical patients and their residual galactose metabolism capacity may prevent them from developing long-term complications, but as they are still young (below nine years of age) a careful long-term follow up is warranted.

The inclusion of the variant patients in our cohort resulted in a number of correlations. Firstly, we found a significant correlation between age and GI, which was not found in our cohort of classical phenotype patients. Secondly, we found a clear correlation between GI and Gal-1-P levels, which is mainly due to the inclusion of the variant patients with higher residual galactose metabolism in fibroblasts and significantly lower Gal-1-P levels when compared to classical phenotype patients. Thirdly, the GI was negatively correlated with whole body galactose oxidation capacity, which can be attributed to the inclusion of variant patients who demonstrated lower GI levels and higher levels of whole body galactose oxidation than classical phenotype patients. Interestingly, within the group of classical phenotype patients with highly variable clinical outcomes, we found no correlation between GI and both Gal-1-P levels and whole body galactose oxidation. In the classical phenotype patients, whole body galactose oxidation was consistently in the low range whereas the GI levels varied considerably. The differences between residual galactose metabolism in fibroblasts and whole body galactose oxidation might be attributed to organ specific GALT activity, which has been observed in an animal model [19]. The same study also demonstrated an age-dependent effect in different tissues. The results of the whole body galactose oxidation study indeed demonstrated significantly higher galactose oxidation capacity in younger patients, also within the group of classical phenotype patients. In the current study, GI levels were not affected by age within the group of classical phenotype patients. Therefore both age and organ specific GALT activity may explain the differences between whole body galactose oxidation results and GI levels.

The finding that the variant patients demonstrated higher residual galactose metabolism in fibroblasts, lower Gal-1-P levels and possibly better clinical outcomes supports our hypothesis that differences in clinical outcomes are caused by differences in residual galactose metabolism capacity. The fact that we did not find this correlation in classical phenotype patients may be due to our small cohort or may suggest that the differences in clinical outcome in this subgroup may be attributed to other factors than residual galactose metabolism such as modifying genes.

There may be a threshold value for residual galactose metabolism in fibroblasts above which clinical outcome improves, but this could not be determined based on the results of this study.

A remarkable result are the low GI levels in our homozygous p.Ser135Leu patients with a poor intellectual outcome. Both patients also demonstrated whole body galactose oxidation in the control range and lower Gal-1-P levels than classical phenotype patients. Therefore, the question remains whether their poor intellectual outcome might be the result of the late initiation of dietary treatment as a comparable intellectual outcome has been reported in another late diagnosed homozygous p.Ser135Leu patient [12,20].

All variant patients were identified and treated early and none of them demonstrates long-term complications at this moment. Possibly their GI level provides sufficient galactose metabolism to protect against long-term complications. Based on the results of this study, the whole body galactose oxidation capacity and the undetectable Gal-1-P levels in NBS detected variants, some variant patients might even benefit from a less strict diet. Before dietary changes are implemented, the clinical outcomes of the variant patients need further follow-up and future studies into dietary relaxation are warranted.

4.1. Limitations

After the exclusion of outliers, the GMP measurements demonstrated an acceptable intra-assay variation, but the inter-assay variation was considerable especially in the group of classical phenotype patients. Despite additional analyses, a clear explanation for the variation could not be determined. The fluctuation in GMP measurements between experiments may indicate that differences in culture and/or incubation conditions influence the GMP measurements.

As previously described, the current method for GMP measurements is limited as it is not able to differentiate [$^{13}C_6$]-UDP-galactose from [$^{13}C_6$]-UDP-glucose and [U^{13}C]-Gal-1-phosphate from [U^{13}C]-glucose-1-phosphate [16]. Considering only labeled substrates are measured which have to pass the GALT enzyme first, this is only a small limitation. The question remains whether the observed differences in GI within the group of classical phenotype patients might be attributed to alternative disposal pathways.

5. Conclusion

The GI measured by GMP in GALT deficient fibroblasts distinguished patients with classical phenotypes from NBS detected variants and homozygous p.Ser135Leu patients. In the classical phenotype patients, the GI was not able to differentiate between patients with a poor and normal clinical outcome. The data of the NBS detected variants support our hypothesis that a higher residual galactose metabolism may result in a more favourable clinical outcome. Further research is needed to enable individual prognostication and treatment in all CG patients.

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Competing interest statement

Mendy M. Welsink-Karssies, Michel van Weeghel, Hyung L. Elfrink, Mirian C.H. Janssen, Kent Lai, Janneke G. Langendonk, Esmee Oussoren, Jos P.N. Ruiter, Eileen P. Treacy, Maaike de Vries and Sacha Ferdinandusse declare they have no conflict of interest. Carla E.M.

Hollak is involved in premarketing studies with Sanofi, Protalix and Idorsia in the field of lysosomal storage disorders. She reports no conflicts of interest in relation to the current study. Annet M. Bosch was member of an advisory board of Biomarin.

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Appendix A. Supplementary data

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