Multi-omics in classical galactosemia: Evidence for the involvement of multiple metabolic pathways

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Abstract
Classical galactosemia (CG) is one of the more frequent inborn errors of metabolism affecting approximately 1:40,000 people. Despite a life-saving galactose-restricted diet, patients develop highly variable long-term complications including intellectual disability and movement disorders. The pathophysiology of these complications is still poorly understood and development of new therapies is hampered by a lack of valid prognostic biomarkers. Multi-omics approaches may discover new biomarkers and improve prediction of patient outcome. In the current study, (semi-)targeted mass-spectrometry based metabolomics and lipidomics were performed in erythrocytes of 40 patients with both classical and variant phenotypes and 39 controls. Lipidomics did not show any significant changes or deficiencies. The metabolomics analysis revealed that CG does not only compromise the Leloir pathway, but
also involves other metabolic pathways including glycolysis, the pentose phosphate pathway, and nucleotide metabolism in the erythrocyte. Moreover, the energy status of the cell appears to be compromised, with significantly decreased levels of ATP and ADP. This possibly is the consequence of two different mechanisms: impaired formation of ATP from ADP possibly due to reduced flux though the glycolytic pathway and trapping of phosphate in galactose-1-phosphate (Gal-1P) which accumulates in CG. Our findings are in line with the current notion that the accumulation of Gal-1P plays a key role in the pathophysiology of CG not only by depletion of intracellular phosphate levels but also by decreasing metabolite abundance downstream in the glycolytic pathway and affecting other pathways. New therapeutic options for CG could be directed towards the restoration of intracellular phosphate homeostasis.

**KEYWORDS**
galactose-1-phosphate, galactosemia, GALT-deficiency, lipidomics, metabolomics, multi-omics

## 1 | INTRODUCTION

Classical galactosemia (CG, OMIM 230400) is caused by a deficiency of the enzyme galactose-1-phosphate uridylytransferase (GALT, EC 2.7.7.12), affects approximately 1:40,000 people, and has an unexplained high variability of outcomes and lack of a prognostic biomarker. Infants with CG may develop a life-threatening disease after ingestion of galactose from breastmilk or infant formula. Elimination of galactose from the diet is lifesaving and to ensure a timely diagnosis and treatment, CG is frequently included in newborn screening (NBS) programs. Currently, the only available treatment option is a lifelong galactose-restricted diet. In spite of early diagnosis and treatment many patients suffer from long-term complications. In our large cohort of patients, we previously reported cognitive impairment in 62% of patients, neurological abnormalities in 47% of patients and impaired fertility in 71% of females. Importantly, strikingly different outcomes have been observed in siblings with the same genetic variations which cannot be explained by illness in the newborn period or age at start of the diet. The pathophysiology of the long-term complications in CG is still poorly understood and prognostic biomarkers are lacking. The continuous endogenous production of galactose results in the persistent elevation of metabolites, such as galactose-1-phosphate (Gal-1P). Gal-1P is considered to have toxic effects and has been demonstrated to affect glycoprotein homeostasis. Indeed N-glycan abnormalities have been reported in CG patients. In our cohort, the most recently measured Gal-1P levels allowed us to discriminate between classical patients (severe GALT deficiency) and variant patients identified by newborn screening with higher residual GALT activity and better outcomes, but not between classical patients with a poor versus normal clinical outcome. Furthermore, differences in outcome in classical patients with a severe GALT deficiency in our cohort did not correlate with other parameters including in vivo galactose oxidation, in vitro galactose oxidation, and IgG galactosylation.

Major technological advances in metabolomics have resulted in the ability to analyze vast numbers of metabolites in patients’ cells and body fluids simultaneously. Metabolite analysis of small molecules (metabolomics) and complex lipids (lipidomics) may result in better understanding of the underlying disease mechanisms and improve patient prognostication with validated biomarkers. In CG, where many small molecules are involved including known metabolites such as galactonate and galactitol this is particularly relevant. Since galactose plays a central role in metabolism being involved in multiple different pathways, a full analysis of the metabolome may well shed new light on CG. Until now, the only published metabolomics study in CG patients evaluated plasma samples only. It was demonstrated that not only the Leloir pathway is affected in CG, but that other metabolic pathways including amino acid metabolism and mitochondrial function are involved as well.

As galactose metabolism takes place in virtually all cells including erythrocytes, and the most frequently used monitoring parameter for CG (Gal-1P) is measured in erythrocytes, we performed semi-targeted metabolomics and lipidomics in erythrocytes of patients with classical
galactosemia and controls with the aim to obtain new insights into the CG pathophysiology possibly leading to new biomarkers.

2 | METHODS

2.1 | Patients and controls

Metabolomics and lipidomics were performed on erythrocyte lysate samples of 40 CG-patients, stored in the Amsterdam UMC Galactosemia Biobank after informed consent of patients and/or parents was obtained for biobanking and the use of clinical data for research after approval of the local medical ethics committee. All patients with two known pathogenic GALT-variants and/or an erythrocyte GALT enzyme activity below 15% of the reference mean were eligible. Patients with a second diagnosis interfering with outcome and/or without the presence of both outcome measurements (intelligence quotient or neurological examination of movement disorders) were excluded. Forty out of 49 stored patient samples were selected prioritizing adult patient samples, due to the availability of only adult control samples. The patient cohort was grouped in three types of patients based on geno- and phenotype: (1) classical patients with two pathogenic GALT gene variants and deficient erythrocyte GALT enzyme activity (<3.3% of reference mean, the limit of quantitation of the enzyme assay), (2) NBS-detected variant patients with previously unknown geno- and phenotypes and an erythrocyte GALT enzyme activity up to 10% of reference mean, and (3) homozygous p.S135L-patients with residual GALT enzyme activity in other tissues but a GALT deficiency in erythrocytes. In order to investigate a possible relation with clinical outcome, subgroups were formed based on intellectual and neurological outcome. Intellectual outcome was defined as poor (IQ < 85) or normal (IQ ≥ 85) and neurological outcome was based on the presence or absence of a movement disorder. It was decided not to form subgroups based on ovarian (dys)function as this complication is seen in nearly all female patients.

Control samples consisted of 40 erythrocyte lysate samples of healthy adults unrelated to the CG-patients. Informed consent was obtained for collection and use of these samples for the development of new laboratory methods in relation to patient care. Collection of all control samples took place on the same day.

2.2 | Sample preparation

Erythrocytes isolated from venous EDTA blood were washed three times in isotonic saline, counted by routine hemocytometric analysis and frozen at −80°C. For each metabolomics and lipidomics analysis an aliquot of 20 μl erythrocytes lysate was used.

2.3 | Metabolomics

Metabolomics analysis was performed as described previously. In short, a 75 μl aliquot of the following internal standards in water was added to 20 μl of washed erythrocytes: adenosine-15N5-monophosphate (100 μM), adenosine-15N5-triphosphate (1 mM), D3-arginine (100 μM), D3-aspartic acid (100 μM), D4-citric acid (100 μM), 13C1-citrtulline (100 μM), 13C6-fructose-1, 6-diphosphate (100 μM), guanosine-15N5-monophosphate (100 μM), guanosine-15N5-triphosphate (1 mM), 13C6-glucose (1 mM), 13C6-glucose-6-phosphate (100 μM), D5-glutamic acid (100 μM), D5-glutamine (100 μM), 13C3-isoleucine (100 μM), D7-leucine (100 μM), D7-lysine (100 μM), D7-methionine (100 μM), D7-ornithine (100 μM), D7-phenylalanine (100 μM), D7-proline (100 μM), 13C3-pyruvate (100 μM), D9-serine (100 μM), D9-tyrosine (100 μM), D9-valine (100 μM). Subsequently, 40 μl water and 500 μl methanol was added and samples were homogenized with a vortex. The 1000 μl of chloroform was added and samples were thoroughly mixed and centrifuged for 10 min (min) at 20.000 g. The top polar layer was transferred to a new 1.5 ml tube and dried using a MiVac vacuum concentrator at 60°C. The residue was dissolved in 100 μl 6:4 (v/v) methanol/water. Metabolites were analyzed using a Waters Acquity UPLC coupled to a Bruker impact II QTOF mass spectrometer as described previously. Data were analyzed using Bruker TASQ software version 2021b. All reported metabolite intensities were normalized to the hemoglobin concentration (in mg) in each sample, as well as to internal standards with comparable retention times and response in the MS. Metabolite identification has been based on a combination of accurate mass, (relative) retention times and fragmentation spectra, compared to the analysis of a library of standards.

2.4 | Lipidomics

Lipidomics was performed as previously described, with minor adjustments. Briefly: In a 2 ml tube, the following amounts of internal standards dissolved in 1:1 (v/v) methanol: chloroform were added to each aliquot of 20 μl erythrocyte sample: Bis(monoacylglycerol)phosphate (BMP(14:0))2 (0.2 nmol), ceramide-1-phosphate C1P (d18:1/12:0) (0.127 nmol), D7-cholesteryl ester CE(16:0) (2 nmol), ceramide Cer(d18:1/12:0) (0.118 nmol), ceramide Cer(d18:1/25:0) (0.130 nmol), cardiolipin CL(14:0)4
(0.1 nmol), diacylglycerol DAG(14:0)₂ (0.5 nmol), glucose ceramide GlcCer(d18:1/12:0) (0.126 nmol), lactose ceramide LacCer(d18:1/12:0) (0.129 nmol), lysophosphatidic acid LPC(14:0) (0.1 nmol), lysophosphatidylcholine LPE(14:0) (0.1 nmol), lysophosphatidylglycerol LPG(14:0) (0.02 nmol), phosphatidic acid PA(14:0)₂ (0.5 nmol), phosphatidylcholine PC(14:0)₂ (2 nmol), Phosphatidylethanolamine PE(14:0)₂ (0.5 nmol), phosphatidylglycerol PG(14:0)₂ (0.1 nmol), Phosphatidylglycerol PE(14:0)₂ (0.5 nmol), Phosphatidylinositol PI(8:0)₂ (0.5 nmol), phosphatidylserine PS(14:0)₂ (5 nmol), sphinganine-1-phosphate S1P(d17:0) (0.124 nmol), sphinganine-1-glycerol TAG(14:0)₂ (0.5 nmol). The 1.5 ml 1:1 (v/v) methanol: chloroform was added before thorough mixing. Each sample was then centrifuged for 10 min at 14,000 rpm. Supernatant was transferred to a glass vial and evaporated under a stream of nitrogen at 60 °C. The residue was dissolved in 150 μl of 1:1 (v/v) methanol: chloroform. Lipids were analyzed using a Thermo Scientific Ultimate 3000 binary HPLC coupled to a Q Exactive Plus Orbitrap mass spectrometer. For normal phase separation, 2 μl of each sample was injected onto a Phenomenex® LUNA silica, 250 * 2 mm, 5 μm 100 Å. Column temperature was held at 25 °C. Mobile phase consisted of (A) 85:15 (v/v) methanol: water containing 0.0125% formic acid and 3.35 mmol/L ammonia and (B) 97:3 (v/v) chloroform: methanol containing 0.0125% formic acid. Using a flow rate of 0.3 ml/min, the LC gradient consisted of: 10% A for 0–1 min, reach 20% A at 4 min, reach 85% A at 12 min, reach 100% A at 12.1 min, 100% A for 12.1–14 min, reach 10% A at 14.1 min, 10% A for 14.1–15 min. For reversed phase separation, 5 μl of each sample was injected onto a Waters HSS T3 column (150 x 2.1 mm, 1.8 μm particle size). Column temperature was held at 60 °C. Mobile phase consisted of (A) 4:6 (v/v) methanol: water and B 1:9 (v/v) methanol: isopropanol, both containing 0.1% formic acid and 10 mmol/L ammonia. Using a flow rate of 0.4 ml/min, the LC gradient consisted of: 100% A at 0 min, reach 80% A at 1 min, reach 0% A at 16 min, 0% A for 16–20 min, reach 100% A at 20.1 min, 100% A for 20.1–21 min. MS data were acquired using negative and positive ionization using continuous scanning over the range of m/z 200 to m/z 2000. Data were analyzed using an in-house developed lipidomics pipeline written in the R programming language (http://www.r-project.org). All reported lipids were normalized to corresponding internal standards according to lipid class and normalized to the hemoglobin concentration (in mg). Lipid identification has been based on a combination of accurate mass, (relative) retention times and the injection of relevant standards.

### 2.5 Statistical analysis

Identified metabolites were classified according to the Human Metabolome Database (HMDB). Lipids were identified using an in-house database of lipids from various lipid classes, with matching ion mass and expected retention times. To identify discriminatory metabolites and their importance for the difference between patients and controls and/or two patient groups, several statistical tests were used. Orthogonal partial least squares regression with Discriminant Analysis (OPLS-DA) was used to calculate Variable Importance in Projection (VIP) scores, which were used to estimate the importance of each metabolite in the differentiation between the groups. A metabolite with a VIP score equal or greater than one was considered important in the given PLS-model.¹⁴ A t-test was performed to assess significant differences in metabolite levels between the combinations of two groups. P-values lower than 0.05 were considered significant. The Bonferroni-HOLM-correction was used to adjust p values for multiple testing. The uncorrected p-values, together with the fold change, are plotted in the volcano plots. For the lipidomics data, effects in individual metabolites were not investigated but the focus was on trends in fatty acid chain length or degree of saturation.

Correlations between the identified metabolites and storage time, between the identified metabolites and intelligence quotient, and between the identified metabolites and Gal-1P were performed on the log-transformed data using Pearson’s correlation. Next, t-tests were used to assess group-differences based on clinical outcome. The Bonferroni-HOLM-correction was used to adjust p-values for multiple testing. All analyses were performed in R.

### 3 RESULTS

#### 3.1 Participants

Samples from 40 patients and 39 control subjects were included in the current study. Patient and control characteristics are shown in Table 1. All patients with the classic phenotype were compliant to the galactose-restricted diet according to the international guideline of 2017⁵ (no restrictions of fruit and vegetables) at the time of blood withdrawal. All variant patients follow a slightly more liberal diet with maximum allowances of 50–100 milliliter milk/day or 1200 milligrams galactose/day. The two homozygous p.S135L-patients both had a late diagnosis and start of diet (respectively 210 and 3860 days). One of the p.S135L-patients was non-compliant to the
diet at the time of blood withdrawal. The patient samples were stored at \(-80^\circ\)C between 836 and 1351 days (\(M = 718.1\), \(SD = 386.0\)) before the start of the omics-analyses. The control samples were stored at \(-80^\circ\)C for 345 days.

### 3.2 Metabolomics

3.2.1 Identifying discriminatory metabolites between patients and controls

Metabolomics analysis of the erythrocyte samples from the 40 patients and 39 controls led to the identification of 115 metabolites (see supplemental file S1). When the results obtained for the entire group of patients and healthy (adult) controls were compared, 38 metabolites reached the threshold of a VIP score equal to or above 1.0 indicating that these metabolites can be considered possibly relevant according to the PLS-DA model (Figure 1A). Of all identified metabolites, 50 were different between patients and controls. The volcano plot of Figure 1B depicts the 22 different metabolites with a \(p\)-value of <0.05 after correction for multiple comparisons either marked in light gray (significantly decreased) or in black (significantly increased) (see supplemental file S2, Table S2.1 for the specific \(p\)-values and VIP-scores of the 38 important metabolites). Most of the important metabolites according to the PLS-DA model also reached the threshold of a VIP score \(\geq 1.0\) in the small subgroup of adult patients with the classical phenotype in comparison to the healthy adult controls (27 out of 38; see supplemental file S2, Table S2.2). Furthermore, after correction for multiple comparisons, the most significant discriminatory metabolites were consistently found in both the entire patient group and a subgroup of adults with the classical phenotype suggesting that there are no effects of age and phenotype. To confirm this, we compared the 27 metabolites with a VIP score \(\geq 1.0\) between adults with the classical phenotype and children with a classical phenotype (\(p = 0.009\)).

To eliminate possible effects of storage time, correlation analyses between the reported metabolites and storage time were performed on the log-transformed data in
the entire patient group (see supplemental file S2, Table S2.1). Only AMP ($r = -0.350$, $p = 0.027$), carnitine (3:0) ($r = 0.430$, $p = 0.006$) and glycerol-3-phosphate (glycerol-3P; $r = -0.324$, $p = 0.041$) were significantly correlated with storage time. This may indicate that AMP, carnitine (3:0) and glycerol-3P are unstable or formed during storage and thus require caution with interpretation. The other reported metabolites did not change with time and were therefore selected for further analysis. After correcting for multiple comparisons, none of the reported metabolites were significantly related. However, due to the stringent nature of the HOLM-correction, the unadjusted p-values were used to select the metabolites for further analysis.

3.2.2 | Involved metabolic pathways that differentiate cases and controls

Based on the discriminatory metabolites, metabolic pathways were investigated for differences between all patients and controls. Three metabolic pathways present in the red blood cell differed significantly.

**Glycolysis**

Figure 2 depicts the results obtained for the different metabolites from the glycolytic pathway. The results show that glucose-6-phosphate (glucose-6P) levels were comparable between patients and controls. The next metabolite or rather group of metabolites labeled “hexose-phosphate (hexose-P)” includes a range of phosphorylated sugars with identical molecular masses which were not differentiated using our current method. Since Gal-1P is one of these sugar phosphates, the cumulative level of sugar phosphates represented as “hexose-P” is elevated. Importantly, the next metabolite in line which is fructose-1,6-diphosphate is significantly lower in patient samples compared to controls. This is in line with the observation that all subsequent metabolites in the glycolytic pathway ranging from glyceraldehyde-3-phosphate (glyceraldehyde-3P) to 1,3-diphosphoglyceric acid, 2-phosphoglyceric acid and phosphoenolpyruvate are all lowered as well, which

![FIGURE 1 Metabolomics results of the comparison between patients and healthy controls. (A) PLS-DA analysis between the patient group and the healthy control group. (B) Volcano plot depicting the metabolomics data of the comparison between patients and healthy controls. Dots represent the significant metabolites between the two groups ($p < 0.05$) after Holm-correction for multiple comparisons.](image-url)
suggests that there may be a reduced flux through the glycolytic pathway. The decrease in metabolite abundance downstream in the glycolytic pathway might be due to the accumulation of Gal-1P either interfering with the step from glucose-6P to fructose-6-phosphate (fructose-6P) or from fructose-6P to fructose-1,6-diphosphate (see Figure 2). Pyruvate levels are higher in patients than in controls without an appreciable effect of storage time. As erythrocytes do not contain mitochondria, pyruvate is the end product of glycolysis that can be converted to lactate with the concomitant conversion of reduced nicotinamide adenine dinucleotide (NADH) to oxidized nicotinamide adenine dinucleotide (NAD$^+$). When discarding one outlier, based on visual exploration of the violin plot of lactate, lactate levels are also higher in patients than in controls ($p = 0.028$).

**Pentose phosphate pathway**

The oxidative phase of the pentose phosphate pathway (PPP) generates reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is the major source of reducing equivalents that are mainly used for the protection of erythrocytes against oxidative injury. The end product of the oxidative phase is ribulose-5-phosphate (ribulose-5P) which can be converted to ribose-5P as required for the generation of phosphoribosylpyrophosphate (PRPP) and thus purine salvage and nucleotide formation in erythrocytes. In Figure 3, the results obtained for the different metabolites from the oxidative phase of the PPP are shown. The PPP starts with glucose-6P of which the levels were comparable between patients and controls. In the first reaction by the rate-controlling enzyme glucose-6P dehydrogenase, gluconolactone-6-phosphate (gluconolactone-6P) is formed together with the cofactor NADPH, which are both significantly elevated in the patient group. The next step catalyzed by the enzyme gluconolactonase produces gluconate-6P which is decreased in the patients’ erythrocytes resulting in an increased gluconolactone-6P/ gluconate-6P-ratio. Furthermore, ribulose-5P (measured in combination with ribose-5P), the end product of the

**FIGURE 2** Results of the metabolomics-analysis for the glycolytic pathway. Violin plots depicting the differences in metabolites from the glycolytic pathway between patients and healthy controls. Significance ($p < 0.05$) is shown before correction for multiple comparisons.
oxidative phase of the PPP is significantly lower in the patient group. Taken together, our findings suggests that there may be a reduced flux through the PPP caused by interference of gal-1P or one or more other metabolites at the level of the enzyme gluconolactonase and/or 6-phosphogluconate dehydrogenase. The other sugar-phosphates of the non-oxidative phase of the PPP showed no significant changes, except for those of the glycolytic pathway which were already described above (decreased glyceraldehyde-3P and increased hexose-P).

**Nucleotide metabolism**

Since erythrocytes do not contain the enzyme glutamine phosphoribosylpyrophosphate (PRPP) amidotransferase (EC 2.4.2.14), purine nucleotides cannot be synthesized via the de novo pathway. Therefore, the synthesis of purine nucleotides in erythrocytes rely entirely on the salvage of purine bases (adenine, hypoxanthine, guanine) and purine nucleosides (adenosine, inosine, guanosine). In patients’ erythrocytes, increased concentrations of Guanosine monophosphate (GMP), IMP and AMP were observed (see Figure 3) which indicates increased salvage of guanine and hypoxanthine by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) to GMP and IMP, respectively and the salvage of adenine to AMP via adenine phosphoribosyltransferase (APRT). The activity of HGPRT and APRT depends strictly on the presence of PRPP, which is produced from glutamine and ribose-5P by PRPP synthetase. Thus, the decreased concentration of ribose-5P might reflect its increase use for the synthesis of PRPP. AMP produced in erythrocytes is phosphorylated to ADP in a reaction catalyzed by adenylate kinase (AK, EC 2.7.4.3). The resynthesis of ATP from ADP involves phosphoglycerate kinase (PGK, EC 2.7.2.3) and pyruvate kinase (PK, EC 2.7.1.40). Those reactions are the only source of ATP in erythrocytes.

Both ATP and ADP were lower in patients’ samples compared to controls, both without storage time effects (see Supplementary material S2, Table S2.1). These alterations in ATP and ADP lead to a lower ATP/ADP-ratio, which points to a decreased energy status within the erythrocytes. Also, the high cellular ratio of AMP (elevated) /ATP (decreased) is a signal that the energy status of the cell is compromised. Unfortunately, AMP is less stable due to the relation with storage time making the
latter implication less reliable. However, AMP does follow the same pattern as the other purine nucleotides, which are not related to storage time, suggesting that storage time cannot fully explain the difference between patients and controls in energy status within the erythrocyte.

3.2.3 | Relation between Gal-1P and metabolites measured in erythrocytes

Currently, the gold standard of biochemical monitoring in CG is Gal-1P in erythrocytes. Gal-1P was measured in 39 out of the total 40 patients in separate blood samples drawn during the same collection as for metabolomics, except for two patients in which Gal-1P was measured 5–6 months earlier. The mean Gal-1P level was 0.38 μmol/gram Hb (range 0–0.72). In the current metabolomics samples, Gal-1P could not be separated from the other hexose-phosphates. The metabolite-group Hexose-P did not correlate with Gal-1P in the entire patient group \( r = 0.26, p = 0.113 \) and in adult patients with the classical phenotype \( r = 0.05, p = 0.851 \). Moreover, the important metabolites according to the PLS-DA model with an VIP score \( \geq 1.0 \) did not correlate with the corresponding Gal-1P levels in both the entire patient group as in the adult patient group with the classic phenotype, except for carnitine (3:0) and carnitine (4:0) in the entire patient group \( r = 0.46, p = 0.004; r = -0.36, p = 0.023 \); results not shown) and glycerol-2P for the adults with classic phenotype \( r = 0.54, p = 0.038 \); results not shown). However, carnitine (3:0), as stated previously, might be affected by storage time. After correcting for multiple comparisons, none of the reported metabolites were significantly related. These results are most likely due to the small range of differences between Gal-1P values in the different patients. The results did not differ after excluding the two patients which Gal-1P was not collected at the same moment as the sample for the omics-analyses (results not shown).

3.2.4 | Identifying discriminatory metabolites between cases with severe and mild outcomes

Intelligence quotient

Almost all patients, except one adult with the classical phenotype, underwent an intelligence assessment. The mean IQ of the entire patient sample was 77.7 (range 45–103). In order to assess the predictive value of metabolomics for clinical outcome, exploratory analyses were performed. Correlation analyses between IQ and the reported metabolites did not reach significance after correcting for multiple comparisons (data not shown). Between the subgroups of classical patients with an IQ \( \geq 85 \) and classical patients with an IQ < 85, 21/38 metabolites reached the threshold of a VIP Score \( \geq 1.0 \) (see supplementary material S2, Table S2.4). Only three metabolites differed significantly between the two groups: ATP (VIP 2.183, \( p = 0.003 \)), ADP (VIP 2.096, \( p = 0.005 \)) and adenine (VIP 1.753, \( p = 0.003 \)). After correction for multiple testing none of the metabolites differed significantly between the two groups indicating that the current identified important metabolites are not predictive for IQ.

Movement disorders

In 32/40 patients a structured neurological examination had been performed. In 17/32 patients a movement disorder (tremor and/or dystonia) was detected. Of the 38 metabolites with a high VIP score discriminatory between patients and controls (\( \geq 1.0 \)), 14 reached the threshold of a VIP Score \( \geq 1.0 \) between patients with or without a movement disorder (see Supplementary material S2, Table S2.5). None of the metabolites differed significantly between these two groups indicating that the current identified important metabolites are not predictive for the presence or absence of movement disorders.

3.3 | Lipidomics

Lipidomics analysis in erythrocytes yielded the annotation of 1453 distinct lipid species (see supplemental file S3). There were no significant differences between patients and controls in the glycosylated lipid classes. As judged from the lipidomics analysis, there were no apparent deficiencies in essential fatty acids in patients (data not shown).

4 | DISCUSSION AND CONCLUSION

Our study is the first to perform intracellular metabolomics and lipidomics in patients with classical galactosemia. The lipidomics analysis did not reveal any differences between patients and controls in glycosylated or other (non-glycosylated) lipid classes. The results of our metabolomics analysis revealed that CG does not only compromise the Leloir pathway, but also involves other metabolic pathways including the glycolytic pathway, the pentose phosphate pathway, and nucleotide metabolism. Our results confirm the finding of the only
previous metabolomics study in CG that multiple pathways are affected. The earlier metabolomics study reported perturbations in glutathione metabolism, amino acids and mitochondrial pathways, which were not replicated in our study. This may be caused by the different approaches and the fact that metabolomics were performed in plasma and erythrocytes respectively. The most striking finding of our study is the compromised energy status of the erythrocyte, with significantly decreased levels of ATP and ADP, a lowered ATP/ADP ratio and a significant increase in IMP, AMP and pyruvate. We suggest that this is the consequence of two different mechanisms: impaired formation of ATP from ADP because of the decrease in metabolite abundance downstream in the glycolytic pathway and trapping of phosphate in Gal-1P which accumulates in CG which is in line with the finding that many metabolites with significantly decreased levels were phosphorylated metabolites.

Historically, phosphate trapping in CG was first suggested by Kogut and colleagues who reported a decrease in plasma phosphate levels in CG patients after ingestion of galactose. In a yeast model of CG, galactose exposure caused a decrease of intracellular phosphate levels that in turn resulted in decreased activity of the phosphate-dependent enzyme glycogen phosphorylase, which was reversible by increasing intracellular phosphate levels. In these cells also a small decrease in ATP levels was demonstrated which is in line with our current findings. In addition, after deletion of the gene encoding for galactokinase (GALK, EC 2.7.1.6) preventing Gal-1P formation, no decrease in phosphate content was seen, which supports the phosphate trapping hypothesis. Oh and colleagues demonstrated absence of product inhibition of Gal-1P on GALK activity in GALT-expressing HEK293T and 143B cells indicating that there is an ongoing production of Gal-1P. They did not show an inhibitory effect of Gal-1P on glycolysis after exposure to galactose, however, which is in contradiction with our observation that from fructose-1-phosphate onward, all subsequent metabolites in the glycolytic pathway were lowered in patients in comparison to healthy controls. Again, the decrease in metabolite abundance downstream in the glycolysis could of course still simply be the result of reduced availability of phosphate.

In literature there has been recent discussion on the truly pathogenic role of Gal-1P in the pathophysiology of CG. While Gal-1P levels measured in bloodspots of neonates upon newborn screening did discriminate between variant patients with higher residual enzyme activities and better outcomes compared to classical patients, no apparent correlation between Gal-1P levels and clinical outcome was found in case of classical patients. In the current study, the measured value of Gal-1P itself was not related to the detected metabolites which could be explained by the small differences in Gal-1P levels between the patients following a galactose-restricted diet. It may well be that it is not the precise extent to which Gal-1P is elevated, but rather that the decrease in metabolite abundance downstream in the glycolysis pathway, suggesting a reduced flux, and the severity of phosphate depletion are the most important pathophysiological factors in CG. Our findings are in line with the notion that the pathophysiology in GC is not only from depletion on intracellular phosphate levels but also by inhibiting flux though the glycolytic pathway and its effect on other pathways.

New therapeutic options should focus on intracellular phosphate homeostasis, for example by preventing the accumulation of Gal-1P though GALK inhibition. Already in 2002, we suggested galactokinase inhibition as an effective treatment for CG, and much effort is made worldwide to develop this therapeutic option. Clinical trials would benefit from a clear predictive biomarker that parallels the disease burden and ideally indicates the phenotypic course. However, the above-described complex pathophysiological process of CG makes it difficult to find this prognostic biomarker, as implicated by our findings that none of the detected metabolites were predictive for intellectual- and neurological outcome and our previous finding that the most recent Gal-1P also was not predictive for clinical outcome.

4.1 Limitations

The current, intracellular study was performed in erythrocytes, which do not contain a nucleus, mitochondria and other organelles. For the lipidomics, we carefully looked at the sphingolipids, especially those containing one or more hexoses (hexosyl/dihexosylceramides), but there was no significant effect in this cohort. As erythrocytes are cells that have lost many organelles involved in (sphingo)lipid metabolism (endoplasmic reticulum, Golgi) lipid effects caused by GALT deficiency may be less apparent than in, for instance, hepatocytes which have all organelles and active lipid metabolism. For the metabolomics, it is probable that not all metabolites involved in the pathophysiological process of CG were detected in this cell type. In addition, the present study evaluates the metabolites within the erythrocytes in a retrospective manner representing one screenshot of the metabolome. Though this form of (static) metabolomics is able to provide insight into the levels of specific metabolites at a certain time point, effects on the flux within the metabolic pathways are deduced from these levels, but are not objectified. Performing metabolic flux analysis in fresh erythrocytes would strengthen our findings...
suggestions the reduced flux through the glycolytic pathway and the phosphate trapping within Gal-1P but are experimentally challenging.

Lastly, unmatched controls were included in the current study. Only adult controls were included even though both pediatric and adult patients participated. Despite possible differences we could not find indications that there were differences between the pediatric and adult patients. Moreover, only information on the diet of patients with CG could be provided. Information regarding the diet of controls was not available. However, the controls metabolize galactose very rapidly and will always have undetectable Gal-1P-levels irrespective of diet. Therefore, both potential limitations due to the usage of unmatched controls are unlikely to affect the conclusions drawn.

In conclusion, this intracellular omics-study highlights the involvement of multiple metabolic pathways in CG. Moreover the compromised energy status of the cell due to the decrease in metabolite abundance downstream in the glycolytic pathway and the trapping of phosphate within Gal-1P are important factors in the pathophysiology of CG leading to new possibilities in the development of therapies for CG.

AUTHOR CONTRIBUTIONS
Merel E. Hermans contributed to the data analysis and interpretation, drafted the initial manuscript and critically revised the manuscript. Michel van Weeghel and Frédéric M. Vaz contributed to the design of the study, the execution of experiments, data analysis and interpretation, preparation of the manuscript and critically revised the manuscript. Sacha Ferdinandusse, André B. P. van Kuilenburg, Mirjam M. C. Wamelink and Ronald J.A. Wanders contributed to the data interpretation, preparation of the manuscript and critically revised the manuscript. Carla E.M. Hollak, Hidde H. Huidekoper and Mirian C. H. Janssen contributed to the data collection and critically revised the manuscript. Mia L. Pras-Raves contributed to the data analysis and interpretation, preparation of the manuscript and critically revised the manuscript. Mendy M. Welsink-Karsssies contributed to the data collection and critically revised the manuscript. Annet M. Bosch contributed to the funding of the study, the design of the study, the data collection, data analysis and interpretation, drafted the initial manuscript, critically revised the manuscript and serves as guarantor for the article. All authors approved the final manuscript for submission.

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CONFLICT OF INTEREST
Merel E. Hermans, Michel van Weeghel, Frédéric M. Vaz, Sacha Ferdinandusse, Hidde H. Huidekoper, Mirian C. H. Janssen, André B. P. van Kuilenburg, Mia L. Pras-Raves, Mirjam M. C. Wamelink, Ronald J.A. Wanders and Mendy M. Welsink-Karsssies declare that they have no conflict of interest. Carla E.M. Hollak declares that she is involved in premarketing studies of Sanofi, Protalix and Idorsia. Annet M. Bosch declares that she received a speakers fee from Nutricia and has been a member of advisory boards for Biomarin.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT
The local medical ethics committee of the Amsterdam UMC approved the biobanking and the usage of clinical data of the patient samples, and the collection and usage of the control samples for research.

PATIENT CONSENT
All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975. The corresponding author confirms that written informed consent has been obtained from all patients, their parents or their legal representatives, and all healthy controls, for biobanking and the use of (clinical) data for research after approval of the local medical ethics committee of the Amsterdam UMC.

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REFERENCES


SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.