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Galactose-1-Phosphate and Galactitol in Red Blood Cells of Galactosemia Patients

Shimaa M. Hamad*[1], Hala H. El-Said[1], Mohammed A. Khedr[2], Ashraf Y. Elfert[1], Gihan A. Sobhy[2], Samar I. Ghanem[1]

Departments of [1]Clinical Biochemistry and Molecular Diagnostics,

[2]Pediatric hepatology, National Liver Institute, Menoufia University, Egypt

***Corresponding author:** Shimaa Mohammed Hamad, **Email:** shimaahamad2014@yahoo.com, **Mobile:** (+20)1019245060

ABSTRACT

Background: Galactose metabolites, like galactitol and galactose-1-phosphate (G-1-P), can accumulate in galactosemia patients on treatment, however it is yet unknown how these metabolites relate to the clinical outcome.

Objectives: The aim of the current work was to measure red blood cells (RBCs) G-1-P and galactitol in healthy and galactosemic infants and children receiving treatment and to demonstrate their effects on clinical outcomes.

Patients and methods: The current study included 20 galactosemia patients, 30 patients suspected for galactosemia and 40 healthy controls of matched age and gender. Complete history taking, physical and ophthalmological examination, and abdominal ultrasound were performed. Liver function tests and hemoglobin concentration were tested. RBCs G-1-P and galactitol were quantified using a novel Ultraperformance Liquid Chromatography- Tandem Mass Spectrometry method.

Results: Treated galactosemia patients showed significant increase in RBCs G-1-P and galactitol in comparison with healthy controls ($p < 0.001$). Significant age-related decrease of these metabolites was found. RBCs galactitol was significantly correlated with albumin and ALT. Significant increase of both metabolites was found in patients with hepatomegaly. No significant correlations between these metabolites and Z score of weight and height were found. In suspected patients' group, RBCs galactitol showed significant increase in comparison with age comparable controls. Two cases from the suspected cases (7%) diagnosed classic galactosemia showed highly elevated levels of RBCs G-1-P and galactitol despite starting a galactose-free diet a few days ago.

Conclusion: This study's findings imply that galactose metabolic status can be evaluated in galactosemic patients using RBCs galactitol and G-1-P and these can be used to assess liver functions.

Keywords: Galactosemia; RBCs G-1-P; RBCs galactitol; UPLC/MS/MS.

INTRODUCTION

Galactosemia is an inherited disorder of carbohydrate metabolism. In Egypt, an incidence ranging from 1:1794 to 1:3000 was reported in previous studies [1, 2, 3]. Countries created neonatal blood screening programs for galactosemia ensure that treatment is started as soon as possible. A galactose-restricted diet is lifesaving early in life, but it doesn't prevent complications, like cognitive impairment, speech problems, motor disturbances and learning disabilities [3].

Patients with any type of galactosemia on treatment aren't truly free from galactose intoxication, due to galactose hidden in nondairy foodstuffs, and endogenous synthesis from UDP-glucose and glycoconjugates turnover naturally occurring. Chronic complications of variable degrees are seen in classic galactosemia and the generalized form of epimerase deficiency. Pathophysiology of these complications is complex and not yet fully understood [4, 5, 6].

G-1-P was assumed to exert inhibitory activity on several enzymes involved in glucose metabolism and glycosylation. Galactitol produced via alternative pathway for catabolism of excess galactose isn't metabolized and builds up in lens fibers and other tissues causing intracellular osmotic and oxidative damage [4, 7]. In clinical practice, measuring RBCs G-1-P and/or urine galactitol concentrations is important for monitoring galactosemic patients receiving a lactose-free diet [8, 9, 10].

The original enzymatic approaches for measurement of galactose metabolites were difficult and time consuming. Chromatography based techniques were developed.

The aim of the current work was to measure RBC G-1-P and galactitol in healthy and galactosemic infants and children receiving treatment and to demonstrate their effects on clinical outcomes.

MATERIALS AND METHODS

The current study included 20 galactosemia patients, and 30 patients suspected for galactosemia, attending at Department of Pediatric hepatology, National Liver Institute, Menoufia University. This study was conducted between January 2021 and June 2022.

Three groups were enrolled in the study: group 1; patients with galactosemia on treatment (n=20), group 2; patients suspected for galactosemia (n=30) and group 3; healthy controls of matched age and gender (n=40). Cases were recruited from outpatient clinics and inpatient wards in Pediatric Department, National Liver Institute (NLI), Menoufia university, Egypt.

Exclusion criteria included patients receiving recent blood transfusion within the last 2-3 months.

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Galactosemia patients were phenotypically diagnosed and improved on lactose free diet. Enzyme activity assay (GALT and GALE) and/ or genotyping were done in 35% of cases. This was due to the high cost of the assays for the patient's family. Four cases were GALT deficient, and four cases were GALE deficient.

Full history taken, clinical and ophthalmological examination of all participants were done. To evaluate growth, Z score of weight and height for age were calculated using WHO growth charts for subjects < 5 years and CDC growth charts for those ≥ 5 years.

Non-fasting peripheral blood samples were collected in EDTA containing tubes. Fresh blood samples were spun at 1200 g for 5 minutes, then plasma and leucocytes were removed. Cold phosphate buffered saline was used to wash RBCs three times. RBCs were stored at - 20 ° C until analyzed to measure RBCs G-1-P and galactitol [7].

Liver function tests (ALT, AST, GGT, total and direct bilirubin, total protein, and albumin) were performed using Beckman Coulter Chemistry Analyzer AU480 (Japan). Hemoglobin concentration was done using a Counter T660 (USA). International Normalized ratio (INR) was calculated from prothrombin time which was measured using reagents provided by Dade-Behring by an STA-Stago Compact CT auto analyzer. Abdominal ultrasound was performed.

Measurement of RBCs G-1-P and galactitol:

Reagents and chemicals

G-1-P dipotassium salt, galactitol (analytical grade; >98% purity) were obtained from Sigma-Aldrich. HPLC grade methanol, acetonitrile and 25% ammonium hydroxide (Fisher Scientific (Loughborough, U.K.)).

UPLC/MS/MS method

RBCs samples were prepared as previously described [11, 12]. The UPLC/MS/MS method was performed with a Waters mass spectrometer with electrospray ionization in negative ion mode in line with a Waters Acquity UPLC system. The elusion was carried out on UPLC BEH amide column (1.7 µm, 100 × 2.1 mm i.d.) from Waters.

5 µL of each sample was injected into the BEH amide column. A gradient formed by mobile phases A (20% acetonitrile in ultrapure water, 0.1% ammonium hydroxide) and B (80% acetonitrile in ultrapure water, 0.1% ammonium hydroxide) dropped linearly from 100% B to 40% B in 5 minutes, followed by 40% B for 1 minute. Following that, 100% B was used to equilibrate the column for 5 minutes in preparation for the next injection. The flow rate was 0.2 mL/min and the temperature of the column was 35 °C.

The mass spectrometer detector conditions were set as previously described [13].Cone voltage was 37 kV for G-1-P and 30 kV for galactitol. Collision energy was 24 eV for G-1-P and 15 eV for galactitol. The method was created using MassLynx V4.1 software (Waters, USA). G-1-P and galactitol were separated and quantified using the ion pairs m/z 259/79[14] and 181.1/89, respectively (Figure 1). The peak areas of G-1-P and galactitol were calculated automatically with Targetlynx software (Waters, USA) and used for quantification.

Method evaluation

Calibration curves of galactitol and G-1-P showed good linearity over a range from 0.1 to 50 µM and 1-200 µM, respectively with linear regression values (R2) > 0.995. Precision and accuracy were calculated for RBCs of control samples spiked with a low (3.125, 1.7 µmol) and a high (100, 50 µmol) G-1-P and galactitol concentrations, respectively. The results for linearity, precision and accuracy of the method were demonstrated in Supplementary 1.

***Ethical Consideration*:**

This study was ethically approved by NLI, Menoufia University Research Ethics Committee (LI IRB #00285/1-3-2022). Written informed consent of all the participants or their legal guardians was obtained. This work has been carried out according to ICH GCP guidelines and applicable local and institutional regulations and guidelines which govern IRB operation for the last 4 years.

Statistical Analysis

With the help of IBM SPSS software version 20, statist ical analysis was carried out (SPSS Inc., CA, USA).

The quantitative data were expressed in median and range (non-parametric). To test differences between two groups, Mann–Whitney U-test was used. Chi- squared test (χ^2) was utilized to study association between two qualitative variables. The Spearman correlation coefficient test (r-test) is a test of significance which is used to study the strength of correlation between non-parametric quantitative variables. The results of r-test can be positive correlation (+) or negative correlation (-).

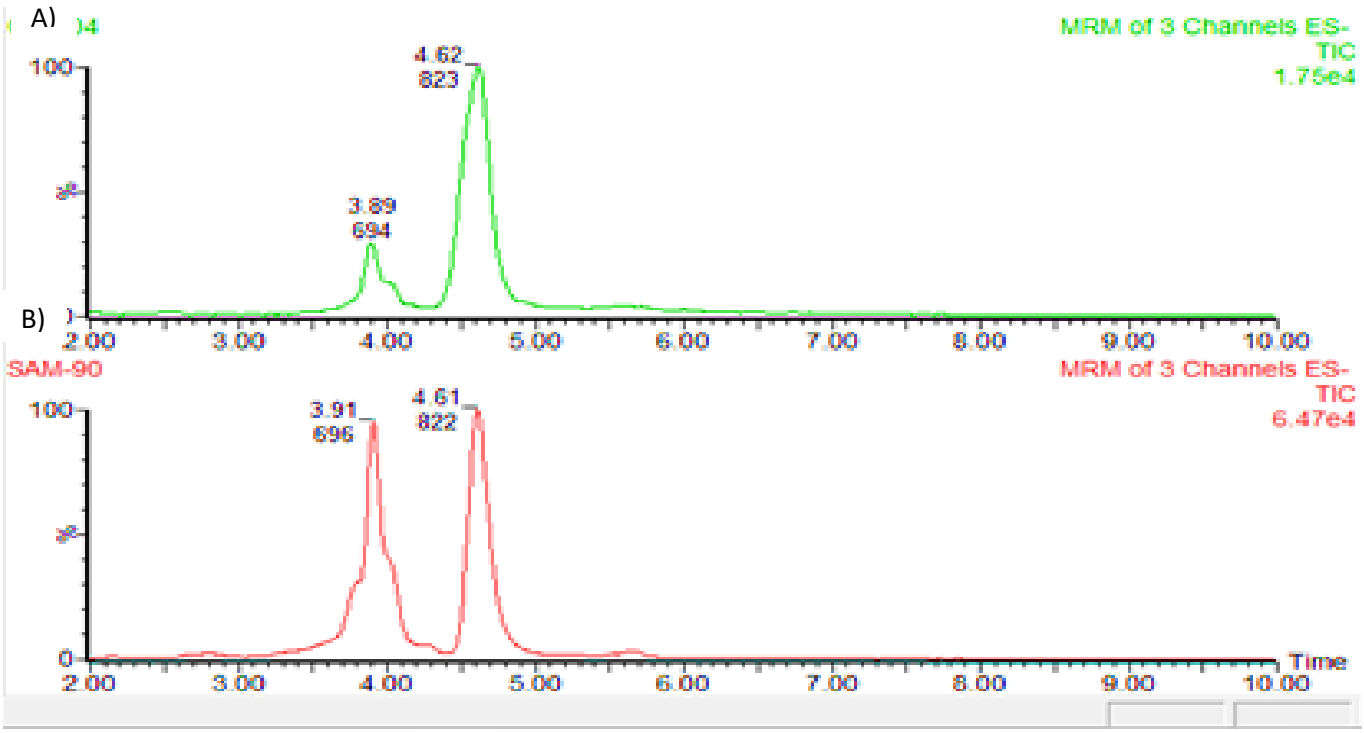

Fig. (1): Multiple reaction monitoring (MRM) chromatograms obtained for chromatographic separation of galactitol and galactose-1- phosphate (G-1-P). A) Control RBC sample supplemented with 12.5 µM of G-1-P and 6 µM of galactitol. B) Galactosemia patient sample containing 306 µM (8 mg/dl) G-1-P and 4 µM galactitol. Retention times: 3.9 min for G-1-P and 4.6 min for galactitol.

RESULT

Table 1, 2 summarize demographic and biochemical findings in studied groups. Age and gender didn't significantly differ between galactosemia and control groups (p- value= 0.198 & 0.652, respectively). 45% of galactosemia patients had positive family history (35% from consanguineous marriage, 10% similar cases and 35% died sibling). 10 % of cases suffered from learning defects and 5% from speech defects. When compared to healthy controls, galactosemia patients' weight and height Z scores were significantly lower (p= 0.001) (60% were ≤ -2 SD) (Table 1).

Six galactosemia patients were shortly diagnosed and initiated lactose free diet (age 3-6 months) with hepatic manifestations. Significant increase in AST, total and direct bilirubin, INR and GGT and significant decrease in total protein and hemoglobin count were found in galactosemia patients in comparison with the control group (Table 1).

Galactosemia patients on treatment showed significant increase of RBCs G-1-P and galactitol when compared with the control group (p- value < 0.001). RBCs G-1-P concentrations (µM) were converted into mg/dl (the widely used unit, 1 mg/dl~ 38.4 µM) [15].

RBCs G-1-P results ranged from 0.03-0.9 mg/dl in the control group. In the treated galactosemia patients, the results ranged from 0.4-8.8 mg/dl and significantly decreased with age (r= - 0.632, p= 0.003).

RBCs galactitol results in healthy subjects ranged between 0.01 and 2 µM. In treated galactosemia patients, RBCs galactitol ranged between 0.15 and 18 µM and significantly decreased with age (r= - 0.48, p= 0.033). Significant correlations were present between RBCs galactitol, and ALT (r= 0.57, p= 0.009) and albumin (r= -0.7, p= 0.001). Weight and height Z scores weren't significantly correlated with any of these metabolites. Significant increase of RBCs G-1-P and galactitol was found in cases with hepatomegaly (p= 0.03 and 0.003, respectively). RBCs galactitol and G-1-P were significantly correlated (r= 0.47, p= 0.04).

Table (1): Demographic and biochemical parameters of galactosemia and control groups

	Galactosemia group (N=20)	Control (N =40)	p-value
Age (months), median(range)	19.5 (3-132)	54 (1-144)	0.593
Gender Male Female	 12(60%) 8(40%)	 19(47.5%) 21 (52.5%)	 0.652
Z- score of weight (≤-2 SD)	12 (60%)	0 (0%)	0.001
Z- score of height (≤-2 SD)	12 (60%)	0 (0%)	0.001
Relevant family history	9 (45%)	0 (0%)	0.001
ALT(IU/L)	22.5 (8-325)	18 (10-32)	0.059
AST(IU/L)	34.5 (2-212)	12 (50-30)	<0.001
Albumin (g/dl)	4 (2-5)	4 (3.5-5)	0.67
GGT (IU/L)	44.5 (15-115)	20 (15-35)	<0.001
Total bilirubin (mg/dl)	1 (0.2-34)	0.5 (0.23-1)	<0.001
Direct bilirubin (mg/dl)	0.2 (0-15)	0.18 (0-0.3)	0.001
Total protein (g/dl)	7 (4-7)	7 (6.5-8)	0.001
INR	1.05 (1-4)	1 (0.8-1.2)	<0.001
Hemoglobin (g/dl)	11(4-13)	12 (11-14)	0.001
RBCs galactitol (µM)	3.01(0.15-18)	0.6 (0.01-2)	< 0.001
RBCs G-1-P (mg/dl)	1.23(0.4 – 8.7)	0.7(0.03-0.9)	< 0.001

ALT, alanine transaminase; AST, aspartate transaminase; GGT, gamma glutamyl transferase; INR, international normalized ratio; G-1-P, galactose-1-phosphate; p- value ≤ 0.05, significant.

Regarding suspected cases, clinical presentations are shown in figure 2. They showed significant increase in ALT, AST, GGT, bilirubin (total and direct) and INR, and significant decrease in albumin, total protein and hemoglobin concentration when compared with age and gender matched group of controls (n=20) (Table 2).

Positive family history was found in 33.3 % of the suspected cases. Significant decrease in Z score of weight and height was found in suspected cases (53.3% and 66.7% were ≤ -2 SD, respectively) (Table 2, Figure 3). RBCs G-1-P levels in the suspected group ranged from 0.3 – 9.2 mg/dl.

They didn't significantly differ from the age comparable control group (p = 0.367). RBCs galactitol in the suspected group ranged from 0.17 to 66 µM. It was significantly elevated in suspected group in comparison with age comparable group of controls (p < 0.001).

Two cases from the suspected group (7%) were diagnosed classic galactosemia and improved on lactose free diet initiated few days ago. Significant positive correlation between RBCs galactitol, and direct bilirubin, total bilirubin and INR was found (r= 0.41, 0.364, 0.395, p= 0.024, 0.048, 0.031, respectively). RBCs G-1-P and galactitol weren't significantly correlated with Z score of weight and height.

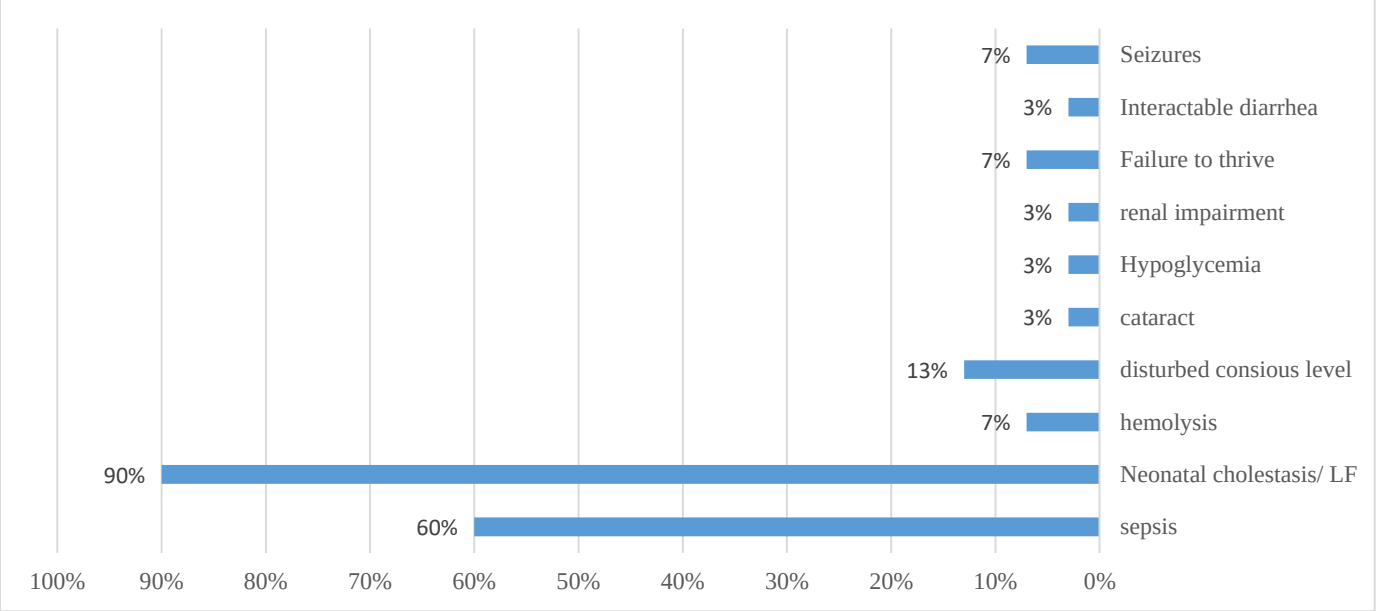

Fig. (2): Clinical presentations in the suspected group.
LF= liver failure

Table (2): Demographic and biochemical parameters of suspected and age comparable group of healthy controls

	Suspected group (N=30)	Control (N =20)	p-value
Age (months), median(range)	4.5 (0.5-36)	5.5 (1-36)	0.199
Gender Male Female	 15(50%) 15(50%)	 7 (35%) 13 (65%)	 0.295
Z- score of weight (≤-2 SD)	16(53.3%)	0 (0%)	<0.001
Z- score of height (≤-2 SD)	20(66.7%)	0 (0%)	<0.001
Relevant family history	10 (33.3%)	0 (0%)	0.004
ALT(IU/L)	81.5 (20-500)	21 (10-32)	<0.001
AST(IU/L)	88.4(30-822)	12 (10-24)	<0.001
Albumin(g/dl)	3.3 (3-4)	4.2 (3.5-5)	<0.001
GGT (IU/L)	55.5 (23-630)	20 (15-35)	<0.001
Total bilirubin (mg/dl)	9.5 (0.5-32)	0.5 (0.23-1)	<0.001
Direct bilirubin (mg/dl)	5 (0.1-23)	0.18 (0-0.3)	<0.001
Total protein (g/dl)	5.8 (4-8)	6.9 (6.5-8)	<0.001
INR	1.4 (0.8-4.5)	1 (0.8-1.2)	<0.001
Hemoglobin (g/dl)	9.8(4-12)	12 (11-14)	<0.001
RBCs galactitol (µM)	2.4(0.17-66)	0.57(0.01-2)	< 0.001
RBCs G-1-P (mg/dl)	0.7(0.3 – 9.2)	0.7(0.03-0.9)	0.367

ALT, alanine transaminase; AST, aspartate transaminase; GGT, gamma glutamyl transferase; INR, international normalized ratio; G-1-P, galactose-1-phosphate; p- value ≤ 0.05, significant.

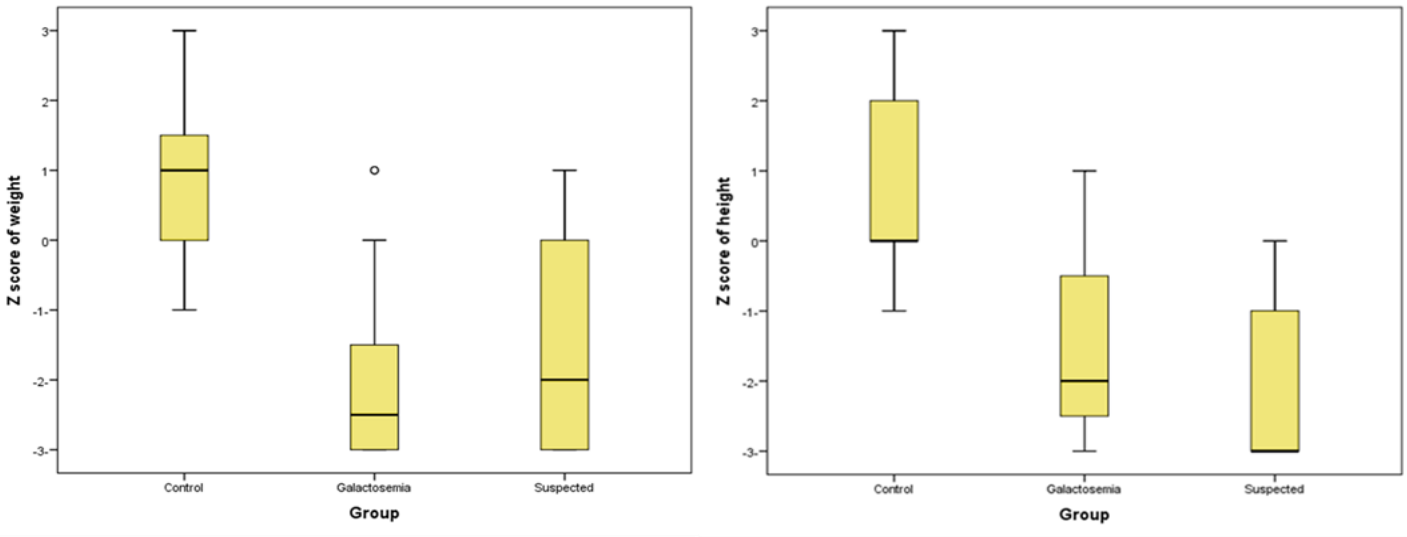

Fig. (3): Simple Boxplot of Z score of weight and height in the studied groups.

DISCUSSION

We presented clinical history and biochemical findings in patients with galactosemia on treatment and patients suspected for galactosemia. We also measured RBCs G-1-P and galactitol using a novel UPLC/MS/MS method which is evaluated in our laboratory with good precision and accuracy.

RBCs G-1-P levels were within normal ranges in healthy newborns and children. The results ranged from 0.03 to 0.9 mg/dl. The findings were consistent with those made by **Cangemi *et al.*** l and Chen *et al*. who reported RBCs G-1-P levels < 1 mg/dl in normal subjects [7, 16]. RBCs G-1-P in treated galactosemia patients ranged from 0.4 to 8.8 mg/dl. In line with these results, **Cangemi *et al.*** found RBCs G-1-P results ranging from 0 to 3.5 mg/dl in treated classic galactosemia (CG) children and adults and from 7.1 to 10.5 mg/dl in CG newborns (0-7 months) [7]. RBCs G-1-P results were decreased with age. This is in line with findings obtained by **Welsink-Karssies *et al.*** [17]. RBCs galactitol results ranged from 0.01 to 2 µM. In treated galactosemia patients, RBCs galactitol results ranged from 0.15 to 18 µM and significantly decreased with age. Accordingly, **Yager *et al***. reported that the range of RBCs galactitol in normal subjects was from 0.29 to 1.29 µM and in treated classic galactosemia patients was from 3.54 to 8.8 µM but with no significant correlation with age [18]. Ficicioglu *et al.* also found that RBCs galactitol levels averaged 6.67 µM in treated galacosemia patients > 1 year with significant age-related decrease [19].

A decreased weight and height (Z score ≤ -2 SD) were found in 60 % of the treated galactosemia patients. Similarly, decreased height and weight has been reported in treated galactosemia children and early teens [20, 21, 22]. Potential risk factors for abnormal growth are either exogenous (dietary restriction, physical activity) or intrinsic factors. Toxic metabolites and aberrant glycosylation of collagen and other proteins are possible intrinsic factors [17, 18].

10% of galactosemia cases included have suffered from learning difficulties and 5% suffering from speech difficulties. Cognitive and speech impairment of varying degrees were frequently reported [17, 22]. One of the primary target organs impacted by galactosemia is the brain. Defective galactolipids and glycoproteins in the white matter and oxidative damage may be involved in the pathogenesis [6]. In this study, RBCs G-1-P and galactitol did not significantly correlate with Z scores for weight and height. Cases suffering from learning and speech defects showed mildly elevated RBCs G-1-P and galactitol. The issue of which biochemical marker which can predict the outcome of galactosemic patients is a long standing and unsolved problem. Welsink-Karssies *et al.* found also no correlation of RBCs G-1-P and long-term outcome in patients with classic galactosemia [17]. However, **Yuzyuk *et al.*** suggested that patients with galactosemia who have higher RBCs G-1-P levels are more prone to experience adverse long-term effects [23].

RBCs galactitol was significantly correlated with ALT and albumin. Significant increase of RBCs galactitol and G-1-P was found in patients with hepatomegaly. Disposal of galactose occurs mainly in liver and galactose toxicity results in liver damage [2, 24 , 25].

RBCs G-1-P and galactitol were also measured in pediatric patients suspected for galactosemia. When compared with age and gender matched healthy infants, there was significant increase in RBCs galactitol but no significant difference in RBCs G-1-P. RBCs galactitol ranged from 0.17 to 66 µM and RBCs G-1-P ranged from 0.3 to 9.2 mg/dl. The highest levels were found in the two cases (7%) which were diagnosed galactosemia and improved on lactose free diet initiated few days ago. In

line with this finding, Fateen *et al*. reported that twenty six out of the 374 high risk Egyptian neonates (7%) were found to be galactosemic [(2)]. RBCs galactitol was significantly correlated positively with INR, total and direct bilirubin. In line with these findings, previous studies reported that liver disease will result in secondary hypergalactosemia which will decrease by time but follow up is important to ensure that it decreases. Lactose free diet may be initiated to prevent the occurrence of cataract [(2, 24, 25)].

CONCLUSION

In conclusion, this study's findings imply that galactose metabolic status can be evaluated in galactosemic patients using RBCs galactitol and G-1-P. Deficient growth is frequently present in galactosemia and liver disease. RBCs metabolites are poorly associated with growth parameters. Liver disease can impair galactose metabolism resulting in transient elevation of galactose and galactitol. RBCs galactitol can be used to assess liver function.

As with many other rare diseases, our findings are based on a limited sample of galactosemia patients with smaller age (long term complications such as sub-fertility in classic galactosemia females aren't yet completely developed and can't be studied). Therefore, more research on a broader group of patients is required to better explain the wide range of clinical outcomes in galactosemia. During patient follow-up, a neurological examination is advised for galactosemia patients.

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Author contribution: Authors contributed equally in the study.

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