Beyond predicting diagnosis: Is there a role for measuring biotinidase activity in liver glycogen storage diseases?

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ABSTRACT

Introduction: Biotinidase synthesis is needed to recycle biotin for essential metabolic reactions. Biotinidase activity is lower than normal levels in advanced liver disease but is higher in hepatic glycogen storage disorders (GSDs), however the cause of this association remains unclear.

Methods: In this study, biotinidase activity was measured in plasma samples from 45 individuals with hepatic GSDs; GSDI (a, b; n = 25) and GSD III (a, b; n = 20), complemented by a chart review to associate biotinidase activity levels with clinical laboratory and imaging findings known to be implicated in these GSDs.

Results: Our findings showed variation in biotinidase activity levels among subjects with GSD I and III; biotinidase activity correlated positively with hypertriglyceridemia in subjects with GSD I (r = 0.47, P = 0.036) and GSD III (r = 0.58, P = 0.014), and correlated negatively with age (r = -0.50, P = 0.03) in patients with GSD III. Additionally, biotinidase activity was reduced, albeit within the normal range in subjects with evidence of fibrosis/cirrhosis, as compared to subjects with hepatomegaly with or without steatosis (P = 0.002).

Discussions: These findings suggest that abnormal lipid metabolism in GSD I and III and progressive liver disease in GSD III may influence biotinidase activity levels. We suggest that a prospective, multi-center, longitudinal study designed to assess the significance of monitoring biotinidase activity in a larger cohort with hepatic GSDs is warranted to confirm this observation.

Take-home message: Altered lipid metabolism and advancing liver fibrosis/cirrhosis may influence biotinidase activity levels in patients with hepatic glycogen storage disease. Thus, longitudinal monitoring of biotinidase activity, when combined with clinical and other biochemical findings may be informative.

1. Introduction

Hepatic glycogen storage diseases (GSDs) are a group of inborn errors of glycogen metabolism and storage. Each of these disorders causes variable degrees of fasting hypoglycemia and hepatomegaly due to an enzymatic defect involving glycogen synthesis or breakdown in the liver. The incidence of both GSD I and III is estimated to be 1:100,000 [1–4]. Patients with GSD I have a deficiency of glucose-6-phosphatase complex (GSD Ia) or glucose-6-phosphate translocase (GSD Ib) enzymes (OMIM #232200 and #232220, respectively). Patients with GSD I (a and b) have fasting intolerance, hypoketotic hypoglycemia, and hepatomegaly due to defective glycogenolysis and gluconeogenesis. They also have secondary hyperlactatemia, hyperuricemia, and hypertriglyceridemia due to shunting of metabolites proximal to the block to alternate pathways, and increased lipolysis [2]. Alternatively, patients with GSD III (a, b) have low debrancher enzyme activity (OMIM #232400), leading to defective glycogenolysis with intact gluconeogenesis. Patients with GSD III usually have ketotic hypoglycemia and hyperlipidemia, and some patients progress to develop chronic liver disease, with signs of fibrosis and cirrhosis [4–6]. Additionally, patients with GSD IIs have progressive muscle disease, with variable degrees of creatine kinase elevations and/or cardiac involvement. Metabolic...
markers such as fasting lactate and uric acid are abnormal in patients with GSD I in poor metabolic control, but usually normal in GSD III; triglycerides are elevated in both disorders, albeit via different mechanisms. Current biochemical markers (lactate, uric acid, and triglycerides) are unreliable as they fluctuate in blood [7] and are influenced by factors such as use of tourniquet for blood draw and composition of the diet. Serum triglyceride (TG) measurement is an important marker to measure and follow over time, as chronic elevations of TGs above 499 mg/dL have been shown to predict an increased risk for hepatic adenomas in GSD I [8]. Biotinidase enzyme is synthesized in the liver and secreted in blood [9], and plays an important role in recycling biotin to conserve its co-factor activity for essential cellular metabolism [10]. Multiple studies have previously demonstrated the usefulness of biotinidase as a diagnostic biomarker in patients with hepatic GSDs, including GSD types I and III; however, the cause of these elevations have not been elucidated [11–16]. In patients with non-GSD hepatic disorders such as hepatitis and advanced liver cirrhosis/failure, biotinidase activity levels have been reported lower than controls [17,18]. Whether biotinidase activity varies among patients with GSD due to metabolic and/or pathological processes inherent to the liver involvement in GSD I and III is clearly unknown. Because the liver is the main source of serum biotinidase synthesis and the main target organ involved in hepatic GSDs, our goal was to assess the utility of measuring biotinidase activity levels in patients with GSD types I and III, with an aim of determining whether metabolic derangements and/or hepatic pathology findings could contribute to the variability in biotinidase activity levels.

2. Patients and methods

2.1. Patients with hepatic GSD I and III and control subjects

This study included 45 individuals with the clinical diagnoses of GSD I (n = 25; 13 F, 12 M) or GSD III (n = 20; 12 F, 8 M), confirmed by low enzyme activity or biallelic pathogenic variants in the genes (G6PC, SLC37A4 or AGL). Reference range for biotinidase activity was determined by analyzing residual samples at Duke Biochemical Genetics Laboratory, with no clinical indication for a hepatic GSD, biotinidase deficiency, or liver/kidney disease, and determined to be unaffected by routine biochemical genetics testing. Healthy volunteers (n = 62) and leftover samples from individuals with Pompe disease (GSD II, OMIM# 232300; Pompe, n = 28), a non-hepatic GSD and lysosomal myopathic disorder without hypoglycemia, were also used as laboratory controls. Individuals included in this study were consented in accordance with the requirements of the Duke University Institutional Review Board (IRB) (GSD III; IRB# Pro00013699, Pro00047556); individuals with GSD I included in this study were evaluated under the natural history study protocol (Pro00000034). Adults were defined as individuals ≥18 years old.

2.2. Biotinidase activity assay

Blood plasma biotinidase assay was developed at Duke Biochemical Genetics Laboratory and used for this study between years 2009–2012. Plasma samples were obtained from EDTA whole blood and were stored at −80 °C. The plasma biotinidase activity was determined using the quantitative chromatographic assay that measures the release of para-aminobenzoate from biotinyl-para-aminobenzoate [19]. The activities were reported as unit (U) of enzyme that catalyzes the transformation of 1 μmol of biotinyl-para-aminobenzoate to para-aminobenzoate per minute, per volume (L) of plasma sample, at 37 °C.

2.3. Clinical laboratory and liver imaging findings

A retrospective chart review of the medical records was performed on individuals consented and included in this study (GSD I and III) to evaluate the relationship between biotinidase activity and both clinical laboratory tests (labs) and imaging findings routinely performed to monitor patients with GSDs. Blood samples and imaging were obtained as part of routine medical management during clinic visits. Liver function tests included analysis of albumin and coagulation parameters (synthetic liver function), bilirubin (excretory liver function), and alanine transaminase (ALT) and aspartate transaminase (AST) (hepatocellular function). Kidney function tests included analysis of creatinine, blood urinary nitrogen (BUN) and microalbumin/creatinine ratio from random urine samples. Metabolic control markers for subjects with GSD I included analysis of lactate, triglycerides (TG), uric acid, and glucose levels. As the presence of 3-methylglutaconic acid (3-MGA) has been observed in some individuals with GSD (Duke; unpublished data) and previously reported in GSD Ib as a sign of abnormal sterol-lipid synthesis [20], urine samples were collected for organic acid analysis (GSD I; n = 12).

To assess the extent of liver involvement, we reviewed study individuals’ medical records for liver imaging results including abdominal ultrasound (US), computerized tomography (CT) scan, and magnetic resonance imaging (MRI), as well as hepatic histopathological reports obtained during liver biopsy where available. For GSD III, histopathological findings were stratified from stages 1 to 4 using the Batts-Ludwig scoring system (stage 1, portal fibrosis; stage 2, perportal fibrosis; stage 3, bridging fibrosis; stage 4, regenerative nodule formation or cirrhosis), as has previously been reported [6]. Liver biopsy results were considered severe for reports demonstrating stage 3 or 4 fibrosis [6].

2.4. Statistical analysis

Descriptive statistical analysis was performed and reported as mean ± standard deviation (SD). The significance of two group comparisons was determined by a two-tailed, unpaired t-test; ANOVA was used for multiple comparisons. We used simple linear regression for correlation studies; a P value ≤0.05 was considered statistically significant.

To evaluate the significance of measuring biotinidase activity in patients with GSD I and III, we used the Receiver Operating Characteristic - Area Under the Curve (ROC-AUC). This was generated by plotting sensitivity versus specificity for subjects with GSD I and III, compared to controls. Statistical significance was achieved when the 95% confidence interval (CI) was above 0.5. Statistical analysis was performed using GraphPad Prism version 9.0.2 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

3. Results

3.1. Biotinidase activity assay performance and control levels

The sample stability and effect of processing on the biotinidase activity was evaluated as part of the method development and validation. The intra-day (n = 6) and inter-day (n = 7, over a 4-week period) coefficient of variation in plasma samples was 3.4% and 12.8%, respectively. After 4 freeze-thaw cycles, the biotinidase activity in plasma showed only 6% decrease. Comparisons of biotinidase activity levels in serum and plasma samples were done on 4 individuals (GSD I, n = 3; GSD III, n = 1), and sample from the same individual showed less than 9% variation between the two samples. The mean biotinidase activity in controls was determined to be 7.2 ± 1.9 U/L (range 1.9–11 U/L, n = 62), and was not influenced by age or gender. The mean biotinidase activity in patients with Pompe disease (non-hepatic GSD) was 8.3 ± 1.6 U/L (range 5.8–12 U/L, n = 28), similar to levels observed in laboratory controls. GSD patient samples (I and III) for biotinidase activity measurement were collected during routine follow-up clinic visits whenever
possible, between 2009 and 2012. The number of times repeat biotinidase activity could be analyzed for each patient is indicated in Tables 1 and 3 for GSD I and GSD III, respectively.

3.2. Clinical findings based on subject demographics

Forty-five patients with GSD (25 F, 20 M) were investigated in this study. The age range was 2–49 years for subjects with GSD I, and 2–58 years for subjects with GSD III. Our cohort included 25 subjects with GSD I (13F, 12 M; 19 with GSD Ia, 6 with GSD Ib) and 20 subjects with GSD III (12F, 8 M; 14 with GSD IIIa, 6 with GSD IIIb). Tables 1 and 2 show biotinidase activity levels, imaging and metabolic parameters measured in GSD I. As shown in Tables 2, 15/25 patients had no adenosomes, while 9/25 had developed adenosomes; age was statistically significant between these two groups (supplementary fig. S1a, P = 0.0141).

Table 1 shows one patient was post-transplant (subject #16, GSD Ia), and 2 patients had renal findings (subject #17, GSD Ia and # 24, GSD Ib). Subject #19, had a kidney transplant only.

Table 3 shows clinical characteristics (age, biotinidase activity and imaging/biopsy findings) of patients with GSD III. Eight out of 20 patients with GSD III showed evidence of fibrosis/cirrhosis on imaging and/or liver biopsy, while 11/20 subjects only showed signs of hepatic megaly and steatosis on abdominal ultrasound without evidence of frank cirrhosis/fibrosis (no biopsies were available). One subject did not have imaging/biopsy findings available during this study period. One individual with GSD IIa (subject # 10) had insulin-dependent type 2 diabetes mellitus and showed liver pathological signs of bridging fibrosis and extensive steatosis.

3.3. Biotinidase activity in patients with GSD I and GSD III

Biotinidase activity was significantly higher in subjects with GSD I (n = 21) and GSD III (n = 19), as compared to unaffected controls (n = 62) and patients with Pompe disease used as non-hepatic GSD controls (n = 28, P < 0.0001; Fig. 1). Four subjects with GSD I were excluded from comparisons of biotinidase activity levels (#16 with post-liver transplant biotinidase value only available, #19 with kidney transplant, and two individuals (#17 and #24) with kidney disease). Individual #17 had mild hepatic megaly with extensive non-obstructive bilateral kidney stones (creatinine 1 mg/dL, GFR within normal limits, with no microalbuminuria), and subject #24 had end stage liver and kidney diseases due to GSD I (MELD score of 20, and on dialysis three times per week, and was awaiting liver and kidney transplant). We also excluded one individual with GSD III due to a known history of insulin-requiring type 2 diabetes mellitus (subject #10).

The mean biotinidase activity level was 7.2 ± 1.9 (1.9–11 U/L) for unaffected controls and 8.3 ± 1.6 (range 5.8–12) for Pompe patients with non-hepatic GSD, respectively. Of note, biotinidase activity levels were higher in subjects with GSD I compared to GSD III (P < 0.01) (Fig. 1). In GSD I samples, the mean biotinidase activity was 16 ± 2.8 U/L (range 10–22 U/L), compared to the mean biotinidase activity of 13.0 ± 3.9 U/L (range 5.7–21.1 U/L) for GSD III samples. In both GSD I and III, biotinidase activity was not affected by gender or GSD subtype (a, b). Biotinidase activity level was not statistically significantly different between children and adults with GSD I. However, in subjects with GSD III, we observed a negative correlation of biotinidase activity with age (r = −0.50, P = 0.03) (Fig. 2A), suggesting that biotinidase activity was comparatively lower in older patients, possibly a reflection of advancing liver disease in older subjects. The Receiver Operating Characteristic - Area Under the Curve (ROC-AUC) for biotinidase activity was 99% for GSD I and 83% for GSD III, as compared to controls (Supplementary figs. S2a and S2b).

In the GSD I cohort, there was no difference in biotinidase activity between subjects with adenosomes compared to subjects without adenosomes. However, subjects with adenosomes were older in age and had higher TG levels compared to subjects without adenosomes (P = 0.0141, and P = 0.0134, respectively) (Supplementary figs. S1a and S1b). Furthermore, TG levels (mean 470, min-max 182–1026 mg/dL) correlated positively with biotinidase activity (r = 0.47, P = 0.036) (Fig. 3A). Additionally, biotinidase activity was significantly higher among subjects with 3-MGA in urine than in subjects without 3-MGA (P = 0.0069) (Fig. 3B). There were no correlations noted between biotinidase activity and kidney functions, liver transaminases, or albumin for patients with GSD I. No statistically significant correlations were observed between biotinidase activity levels and glucose, lactate and uric acid (Table 3) for patients with GSD I. Imaging findings and corresponding biotinidase

### Table 1

| Subjects | Age (years) | Biotinidase activity, average (U/L) | Liver involvement |
|----------|-------------|-------------------------------------|-------------------|
| GSD Ia (n = 25) | | | |
| 1 | 4 | 20.1 (n = 2) | Ultrasound: hepatic megaly, echogenic, steatosis |
| 2 | 5 | 16.1 (n = 2) | Ultrasound: hepatic megaly |
| 3 | 6 | 12.3 (n = 2) | Ultrasound: hepatic megaly, echogenic, steatosis |
| 4 | 8 | 12.5 (n = 3) | Ultrasound: hepatic megaly, echogenic liver |
| 5 | 9 | 13.9 (n = 3) | Ultrasound: hepatic megaly, echogenic, steatosis |
| 6 | 11 | 14.0 (n = 4) | Ultrasound: hepatic megaly, echogenic, steatosis |
| 7 | 13 | 17.1 (n = 2) | Ultrasound: hepatic megaly, echogenic, steatosis |
| 8 | 14 | 16.9 (n = 1) | MRI: hepatic megaly, steatosis, multiple adenoma |
| 9 | 15 | 10.8 (n = 2) | Ultrasound: hepatic megaly |
| 10 | 18 | 12.8 (n = 2) | MRI: hepatic megaly, fatty, multiple adenoma |
| 11 | 18 | 16.7 (n = 1) | N/A |
| 12 | 20 | 21.6 (n = 2) | Ultrasound: hepatic megaly, echogenic, steatosis |
| 13 | 24 | 16.9 (n = 5) | MRI: hepatic megaly, multiple adenomas |
| 14 | 24 | 18.1 (n = 5) | MRI: severe hepatic megaly, steatosis, multiple adenomas |
| 15 | 30 | 15.9 (n = 1) | MRI: hepatic megaly, diffuse steatosis, multiple adenoma |
| 16 | 35 | 6.8 (n = 2) | Post-liver transplant (no level prior to transplant) |
| 17 | 36 | 9.3 (n = 1) | Mild hepatic megaly, extensive non-obstructing bilateral nephrolithiasis |
| 18 | 38 | 15.6 (n = 2) | MRI: hepatic megaly, steatosis, multiple adenoma |
| 19 | 49 | 16.5 (n = 3) | MRI: hepatic megaly, steatosis, multiple adenomas, kidney transplant |

| GSD Ib (n = 6) | | | |
| 20 | 2 | 16.7 (n = 3) | Ultrasound: hepatic megaly, echogenic, coarse |
| 21 | 7 | 17.5 (n = 3) | Ultrasound: hepatic megaly, echogenic, coarse |
| 22 | 9 | 19.8 (n = 5) | Ultrasound: hepatic megaly, echogenic, coarse |
| 23 | 11 | 15.9 (n = 2) | MRI: hepatic megaly, diffuse steatosis, multiple adenoma |
| 24 | 29 | 10.6 (n = 3) | CT: hepatic megaly, portal hypertension, kidney failure |
| 25 | 32 | 12.7 (n = 1) | MRI: hepatic megaly, steatosis, multiple adenomas |

Note: n, number of samples. N/A; not available.
activity levels (Table 1) and metabolic control parameters (with and without adenomas) are shown in Table 2. Three subjects (#16 post-transplant and subjects #17 and #24 with obstructive uropathy and dialysis dependent chronic kidney disease, respectively) had lower biotinidase activity levels (within the control range) compared to other patients with GSD I and higher levels.

In the GSD III cohort, biotinidase activity correlated with hypertriglyceridemia (mean 193, min-max 76–480 mg/dL) (r = 0.58, P = 0.0144) (Fig. 2B). We report a significant decrease in the mean biotinidase activity among subjects with evidence of liver cirrhosis/fibrosis on imaging and/or biopsy, compared to subjects showing only hepatomegaly or steatosis without evidence of liver fibrosis on imaging and/or biopsy, compared to subjects showing only hepatic glycogen storage disorders (GSDs), reliable non-invasive biomarkers that reflect hepatic, biochemical, and pathological changes become a necessity. In this study, we evaluated repurposing the measurement of biotinidase activity in subjects with GSD I and III to assess its relevance as a biomarker for chronic disease. We chose to study patients with GSD I and III because they have distinct biochemical features (especially GSD I) and liver findings. Furthermore, new therapies are currently in different phases of clinical trials.

In summary, using ROC graphs, we show the sensitivity and specificity of biotinidase as a marker in patients with GSD I, and III. Our results support previous findings, showing higher levels of biotinidase activity in patients with GSD I, when compared to GSD III patients. We show that there is more variability in biotinidase activity levels among patients with GSD III than in GSD I. We report that age and biotinidase activity were inversely related in patients with GSD III and evidence of liver pathology on imaging and liver biopsy. We also report that biotinidase activity is higher among patients with GSD Ia who have elevated 3-MGA.

In our study, lower but within “normal control” biotinidase levels were noted in 3/25 patients with GSD I, and 8/20 subjects with GSD III. In three GSD I patients, this was attributed to liver transplant in one patient, and was associated with obstructive kidney disease, and renal failure respectively in two patients. Nevertheless, biotinidase activity is high in the kidneys, and the kidneys play an important role in glycogen metabolism and gluconeogenesis [21]. Of note, patients with type 1 diabetes, who develop renal dysfunction and nephropathy, reportedly lose biotinidase and alanine in urine with advancing kidney disease [22]. Whether kidney disease in patients with GSD I could influence biotinidase activity levels is unknown and cannot be deduced from this limited study.

In individuals with GSD III, we observed significant variations in biotinidase activity levels among eight individuals with advanced liver pathology. Lower biotinidase activity was associated with lower controls. As biomarkers, ROC-AUC of biotinidase activity assay sensitivity was 0.50 in patients with GSD I and 0.50, respectively in two patients. Nevertheless, biotinidase activity is high in the kidneys, and the kidneys play an important role in glycogen metabolism and gluconeogenesis [21]. Of note, patients with type 1 diabetes, who develop renal dysfunction and nephropathy, reportedly lose biotinidase and alanine in urine with advancing kidney disease [22]. Whether kidney disease in patients with GSD I could influence biotinidase activity levels is unknown and cannot be deduced from this limited study.

In individuals with GSD III, we observed significant variations in biotinidase activity levels among eight individuals with advanced liver pathology. Lower biotinidase activity was associated with lower controls.

### Table 2

| Age (years) | Biotinidase activity (U/L) | Lactic Acid (RI: <2.5 mmol) | Triglycerides (mg/dL) (>499, very high) | Uric acid (RI: <6 mg for females, <7 mg for males) | Glucose (RI: 70–130 mg/dL) |
|------------|--------------------------|----------------------------|----------------------------------------|-----------------------------------------------|--------------------------|
| GSD I participants without hepatocellular adenomas (n = 15) | | | | | |
| 2 | 16.7 | 3.0 | 182 | 10.7 | 85 |
| 4 | 20.1 | 6.0 | 764 | 5.6 | 111 |
| 5 | 16.1 | 4.1 | 208 | 5.4 | 92 |
| 6 | 12.3 | 2.6 | 235 | 4.4 | 65 |
| 7 | 17.5 | 3.0 | 185 | 7.2 | 77 |
| 8 | 12.5 | 2.4 | 286 | 5.8 | 97 |
| 9 | 13.9 | 3.6 | 317 | 6.1 | 106 |
| 9 | 19.8 | 7.6 | 427 | 10.4 | 80 |
| 11 | 14 | 4.5 | 272 | 6.1 | 90 |
| 13 | 17.1 | 3.8 | 190 | 7.7 | 96 |
| 14 | 16.9 | 9.7 | 808 | 9.4 | 44 |
| 15 | 10.8 | 3.5 | 394 | 8.9 | 83 |
| 18 | 16.7 | N/A | N/A | N/A | N/A |
| 29 | 10.6 | 6.9 | 270 | 6.0 | 141 |
| 36 | 9.3 | 6.7 | 363 | 10.8 | 88 |
| Mean: 12 (interval: 2–35) | 15.0 (9.3–20) | 4.8 (2.4–9.7) | 350.1 (182–808) | 7.5 (4.4–10.8) | 90.0 (44–141) |

| GSD I participants with hepatocellular adenomas (n = 9) | | | | | |
| 11 | 15.9 | 2.0 | 475 | 6.4 | 102 |
| 18 | 12.8 | 10.0 | 2000 | N/A | N/A |
| 20 | 21.6 | 6.3 | 550 | 7.3 | 61 |
| 24 | 16.9 | 7.0 | 1026 | 7.3 | 60 |
| 24 | 18.1 | 7.4 | 815 | 8.0 | 71 |
| 30 | 15.9 | 5.8 | 717 | 7.4 | 80 |
| 32 | 12.7 | 2.6 | 519 | 7.1 | 151 |
| 38 | 15.6 | 8.4 | 352 | 10.7 | 45 |
| 49 | 19.5 | 8.1 | 684 | 6.8 | 80 |
| Mean: 27 (interval: 11–49) | 16.6 (12.7–21.6) | 6.4 (2.0–10.0) | 793 (352–2000) | 7.6 (6.4–10.7) | 81.3 (45–151) |

Note: Table excluding subject #16 (post-liver transplant, age 35 yrs). RI; Reference Interval. N/A; not available.
enzymatic activity or limit increasing levels in some patients with GSD Ia showed a significant positive correlation between biotinidase activity and TGs. In a recent study by Forny and colleagues (2021), five patients with GSD Ia showed a significant positive correlation between biotinidase and triglycerides, attributed to the increased need for biotin as a cofactor to carboxylases, possibly due to deranged gluconeogenesis and fatty acid synthesis in GSD Ia.

**Table 3**

| Subjects | Age (years) (interval) | Biotinidase activity, average (U/L) | Liver involvement |
|----------|------------------------|-------------------------------------|-------------------|
| GSD III (n = 20) | | | |
| 1 | 2 | 20.0 (n = 1) | Ultrasound: hepatomegaly, steatosis |
| 2 | 3 | 12.5 (n = 1) | N/A |
| 3 | 6 | 21.1 (n = 2) | Ultrasound: hepatomegaly, steatosis |
| 4 | 8 | 12.6 (n = 3) | Ultrasound: hepatomegaly |
| 5 | 10 | 8.5 (n = 2) | Ultrasound: coarse liver, cirrhosis, steatosis |
| 6 | 10 | 9.1 (n = 3) | Ultrasound: hepatomegaly, bridging fibrosis on biopsy |
| 7 | 10 | 17.7 (n = 1) | Ultrasound: hepatomegaly, mild echogenicity |
| 8 | 13 | 13.8 (n = 1) | Ultrasound: hepatomegaly, increased echogenicity |
| 9 | 16 | 15.8 (n = 2) | MRI: hepatomegaly, steatosis, no focal findings |
| 10 | 16 | 19.2 (n = 1) | Stage 3 (bridging fibrosis) on liver biopsy, coarse echogenicity on ultrasound with diffuse steatosis, fatty liver, type 2 diabetes mellitus (insulin requiring) |
| 11 | 35 | 14.7 (n = 2) | MRI: hepatomegaly |
| 12 | 36 | 15.0 (n = 1) | Stage 3 (bridging fibrosis), on liver biopsy, evidence of steatosis, coarse echogenicity on ultrasound |
| 13 | 38 | 10.6 (n = 4) | Stage 4 (regenerative nodule formation, cirrhosis) on liver biopsy |
| 14 | 57 | 10.0 (n = 2) | Stage 4 (regenerative nodule formation, cirrhosis) on liver biopsy |
| GSD IIIb (n = 6) | | | |
| 15 | 10 | 14.8 (n = 2) | Ultrasound: liver normal |
| 16 | 11 | 12.0 (n = 1) | CT abdomen: hepatomegaly |
| 17 | 17 | 11.5 (n = 1) | CT abdomen: hepatomegaly |
| 18 | 41 | 12.8 (n = 4) | Ultrasound: hepatomegaly |
| 19 | 47 | 5.7 (n = 3) | Stage 4 MRI: cirrhosis, fibrosis, dysplastic nodule |
| 20 | 58 | 9.3 (n = 3) | Stage 4 MRI: cirrhosis, fibrosis, steatosis |

Note: 8/20 subjects (shown as bold) had evidence of fibrosis/cirrhosis on imaging and/or liver biopsy. n, number of samples. N/A, not available.

We observed a significant positive correlation between biotinidase activity and TGs. In addition, we report that biotinidase activity was higher in patients with GSD I, who had 3-MGA. Presence of 3-MGA was previously reported in a patient with GSD Ib; the cause of this finding was postulated to be due to alteration in the balance between lipid synthesis and gluconeogenesis, where 3-MGA correlated with elevated levels of Krebs cycle intermediates [20,24,25]. Interestingly, disturbed hepatic mitochondrial abnormalities involving the Krebs cycle have been previously described in a GSD I mouse model and human fibroblasts [26,27].

In patients with GSD III, as children grow through adolescence and adulthood, the metabolic phenotype and liver enzymes appear to “deceivingly improve”, despite worsening muscle and liver disease on imaging and biopsy [2,6,28]; in approximately 15% of adult patients, severe hepatic manifestations are reported [29]. Notably, in our study, patients with GSD III showed lower biotinidase activity levels compared to those with GSD I. These trends were mostly observed in individuals with signs of advanced liver fibrosis and cirrhosis, suggesting that lower biotinidase activity in these subjects may be a marker of worsening liver disease pathology. We also observed a negative correlation between biotinidase activity and age. Thus, we speculate progressive changes that take place in the liver of subjects with advancing age may be causing lower biotinidase activity levels in this group. Of note, four participants with advanced liver disease and relatively lower biotinidase activity levels were in their third to fifth decades of life. Our findings corroborate a previously published report showing low biotinidase activity in individuals with GSD III with evidence of liver cirrhosis, attributing this to a biosynthetic hepatic dysfunction [14]. Taken together, these findings suggest that seemingly hepatomegaly and steatosis are associated with high biotinidase levels, similar to what is observed in patients with GSD I, while severe fibrosis and cirrhosis may lower levels of biotinidase activity.

Recent report by Forny and colleagues (2021) of two patients with GSD Ia and GSD IV, using gene expression studies, showed increased levels of phosphoenolpyruvate carboxykinase (PEPCK) and fructose-1,6-bisphosphatase, indicating increased flux through the gluconeogenesis pathway; these patients were also noted to have increased biotin.
dependent pyruvate and acetyl-CoA carboxylases, needed to provide substrates for gluconeogenesis and fatty acid synthesis [30]. Our findings of a relationship between biotinidase activity and triglycerides in a larger number of GSD I patient, and in patients with GSD III further corroborate the findings of Forny and colleagues.

In conclusion, variations in levels of biotinidase activity in patients with hepatic GSDs may be a sign of metabolic (disturbed gluconeogenesis and fatty acid synthesis) and pathological (advancing fibrosis and cirrhosis) processes that influence biotinidase activity levels. This variability may reduce its use as a diagnostic biomarker, as it may become unreliable in conditions associated with advanced liver and/or kidney diseases. Thus, longitudinal monitoring of biotinidase levels may be beneficial to assess variation in biotinidase levels from baseline over time. It would also be interesting to assess the newer imaging techniques, like fibroscans or liver ultrasound with elastography, to see whether they are advantageous in patients with GSD compared to ultrasound and MRI (standard of care), and if there was any association with biotinidase levels. The major limitation of this study is that it was a cross sectional observational and retrospective study, with no systematic longitudinal data collected. There is a need for prospective multi-centered longitudinal study designed to address the exact role of biotinidase activity levels in disease monitoring and measure disease outcomes.

**Contributions**

A El-Gharbawy; collected data from medical records, critically reviewed and re-analyzed data, conceptualized study, revised and assisted in drafting the initial and the final manuscript.

AA Tolun; developed and validated the biotinidase activity assay, performed enzyme analysis and collected data, analyzed data, and conceptualized the study, drafted the initial manuscript, and revised the final manuscript.

CA Halaby; collected data from medical records, contributed to the

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**Fig. 2.** Biotinidase activities in GSD III.

A) Biotinidase activity in GSD III decreases with increased age (n = 19, r = -0.50, P = 0.03). Activities are reported as U/L, and age in years (yrs).

B) Biotinidase activities correlated with triglyceride (TG) levels in GSD III (n = 17, r = 0.58, P = 0.0144). Activities are reported as U/L, and TG levels in mg/dL. TG levels observed in participants with GSD III were relatively lower (mean 193, min-max 76–480 mg/dL) than levels obtained from those with GSD I (mean 470, min-max 182–1026 mg/dL) (see Fig. 3A).

C) Advanced liver pathology influences biotinidase activity in GSD III. Biotinidase activities were significantly different between participant groups with (n = 7) and without (n = 11) advanced liver pathology (P = 0.0021). Activities are reported as U/L.

No Cirrhosis Group; hepatomegaly with or without steatosis and no cirrhosis. Cirrhosis Group; liver fibrosis and cirrhosis.
TG levels observed in patients with GSD I were much higher (mean 470, min–max 19, 1026 mg/dL) than levels obtained from participants with GSD III (mean 193, min–max 76–480 mg/dL) (see Fig. 2 B).

Activities are reported as U/L. A) Biotinidase activities correlated with triglyceride (TG) levels in GSD I (n = 19, r = 0.47 P = 0.036). Activities are reported as U/L, and TG levels in mg/dL. TG levels observed in patients with GSD I were much higher (mean 470, min-max 182–1026 mg/dL) than levels obtained from participants with GSD III (mean 193, min-max 76–480 mg/dL) (see Fig. 2B).

B) When increased, biotinidase activity was higher in participants with GSD I that had elevated 3-methylglutaconic acid (3-MGA) identified by qualitative urine organic acid analysis (with 3-MGA, n = 5) versus those that did not show elevations (without 3-MGA, n = 7) (P = 0.0063). Activities are reported as U/L.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2022.100856.

References

[1] A. Dagli, C.P. Sentner, D.A. Weinstein, Glycogen storage disease type III, in: M. P. Adam, H.H. Ardinger, R.A. Pagon, S.E. Wallace, L.J.H. Bean, K. Stephens, A. Amemiya (Eds.), GeneReviews(R)), 2016. Seattle (WA).
[2] P.S. Kishnani, S.L. Austin, J.E. Abdenur, P. Arn, D.S. Bali, A. Boney, W.K. Chung, A. I. Dagli, D. Dale, D. Koepke, M.J. Somers, S.B. Wechsler, D.A. Weinstein, J. L. Woldendorf, M.S. Watson, American College of Medical, G American College of Medical Genomics, Diagnosis and management of glycogen storage disease type I: a practice guideline of the American College of Medical Genetics and Genomics. Genet. Med. 16 (2014), e1.
[3] P. Laforet, D. Weinstein, G. Smit, The Glycogen Storage Diseases and Related Disorders, Springer, Heidelberg, 2012.
[4] P.S. Kishnani, S.L. Austin, P. Arn, D.S. Bali, A. Boney, L.E. Case, W.K. Chung, D. M. Desai, A. El-Gharbawy, R. Haller, G.P. Smith, A.D. Smith, L.D. Hobson-Webb, S. B. Wechsler, D.A. Weinstein, M.S. Watson, Acmg, glycogen storage disease type III diagnosis and management guidelines, Genet. Med. 12 (2010) 446–463.
[5] E. Demo, D. Fruh, M. Gottfried, J. Koepke, A. Boney, D. Bali, Y.T. Chen, P. S. Kishnani, Glycogen storage disease type III-hepatocellular carcinoma a long-term complication? J. Hepatol. 46 (2007) 492–498.
[6] C.A. Halaby, S.P. Young, S. Austin, E. Stefanescu, D. Bali, L.K. Clinton, B. Smith, S. Pendyal, J. Lipadia, G.R. Schoofler, A.M. Mavir, P.S. Kishnani, Liver fibrosis during clinical ascertainment of glycogen storage disease type III: a need for improved and systematic monitoring. Genet. Med. 21 (2019) 2686–2694.
[7] T.G. Derks, M. van Rijn, Lipids in hepatic glycogen storage diseases: pathophysiology, monitoring of dietary management and future directions, J. Inherit. Metab. Dis. 38 (2015) 537–543.
[8] D.Q. Wang, L.M. Fiske, C.T. Carreras, D.A. Weinstein, Natural history of hepatocellular adenoma formation in glycogen storage disease type I. J. Pediatr. 159 (2011) 442–446.
[9] R.E. Grier, G.S. Heard, P. Watkins, B. Wolf, Low biotinidase activities in the sera of patients with impaired liver function: evidence that the liver is the source of serum biotinidase. Clin. Chim. Acta 186 (1990) 397–400.
[10] J. Hynes, B. Wolf, Biotinidase and its roles in biotin metabolism, Clin. Chim. Acta 255 (1996) 1–11.
[11] C.J. Angaroni, A.N. Giner-Ayala, L.P. Hill, N.B. Guelbert, A.E. Paschini-Capra, R. D. de Kremer, Evaluation of the biotinidase activity in hepatic glycogen storage disease patients. Undescribed genetic finding associated with atypical enzymatic behavior: an outlook, J. Inherit. Metab. Dis. 33 (2010) S289–94.
[12] A.B. Burlina, M. Dermikol, A. Mantau, S. Piovan, L. Grazian, F. Zaccelini, Y. Shin, Increased plasma biotinidase activity in patients with glycogen storage disease type ia: effect of biotin supplementation, J. Inherit. Metab. Dis. 19 (1996) 209–212.
[13] G. Hug, G. Chuck, M. Tsoras, Increased serum biotinidase activity in glycogen storage disease type ia, Pediatr. Res. 35 (1994) 20A.
[14] P. Paesold-Burda, M.R. Baumgartner, R. Santer, N.U. Bohnard, B. Steinmann, Elevated serum biotinidase activity in hepatic glycogen storage disorders–a convenient biomarker, J. Inherit. Metab. Dis. 30 (2007) 896–902.
[15] I.N. Saltik, H. Ozen, N. Kocak, A. Yuce, F. Gurakan, High biotinidase activity in type ia glycogen storage disease, Am. J. Gastroenterol. 95 (2000) 2144.
[16] B. Wolf, C.L. Freehauf, J.A. Thomas, P.L. Gordon, C.L. Greene, J.C. Ward, Markedly elevated serum biotinidase activity may indicate glycogen storage disease type ia, J. Inherit. Metab. Dis. 26 (2003) 805–809.

[17] A. Pabuccuoglu, S. Aydogdu, M. Bas, Serum biotinidase activity in children with chronic liver disease and its clinical significance, J. Pediatr. Gastroenterol. Nutr. 34 (2002) 59–62.

[18] M. Faith, C.E. Eapen, G. Wilfred, J. Ramachandran, M. Jacob, Serum biotinidase is a sensitive and specific biochemical marker of hepatic dysfunction: a preliminary report, Hepatol. Res. 37 (2007) 13–17.

[19] B. Wolf, R.E. Grier, R.J. Allen, S.I. Goodman, C.L. Kien, Biotinidase deficiency: the enzymatic defect in late-onset multiple carboxylase deficiency, Clin. Chim. Acta 131 (1983) 273–281.

[20] L.K. Law, N.L. Tang, J. Hui, C.W. Lam, T.F. Fok, 3-methylglutaconic aciduria in a chinese patient with glycogen storage disease ib, J. Inherit. Metab. Dis. 26 (2003) 705–709.

[21] J.E. Gerich, Role of the kidney in normal glucose homeostasis and in the hyperglycemia of diabetes mellitus: therapeutic implications, Diabet. Med. 27 (2010) 136–142.

[22] E.A. Terentyeva, K. Hayakawa, A. Tanae, N. Katsumata, T. Tanaka, I. Hibi, Urinary biotinidase and alanine excretion in patients with insulin-dependent diabetes mellitus, Eur. J. Clin. Chem. Clin. Biochem. 35 (1997) 21–24.

[23] L.Y. Chiu, P.S. Kishnani, T.P. Chuang, C.Y. Tang, C.Y. Liu, D. Bai, D. Koeberl, S. Austin, K. Boyette, D.A. Weinstein, E. Murphy, A. Yao, Y.T. Chen, L.H. Li, Identification of differentially expressed microRNAs in human hepatocellular adenoma associated with type i glycogen storage disease: a potential utility as biomarkers, J. Gastroenterol. 49 (2014) 1274–1284.

[24] B. Su, R.O. Ryan, Metabolic biology of 3-methylglutaconic acid-uria: a new perspective, J. Inherit. Metab. Dis. 37 (2014) 359–368.

[25] S.B. Wortmann, L.A. Klujiitmans, U.F. Engelke, R.A. Wevers, E. Morava, The 3-methylglutaconic acidurias: what's new? J. Inherit. Metab. Dis. 35 (2012) 13–22.

[26] R.L. Farah, R.A. Sinha, Y. Wu, B.K. Singh, A. Lim, M. Hirayama, D.J. Landau, B. H. Bay, D.D. Koeberl, P.M. Yen, Hepatic mitochondrial dysfunction is a feature of glycogen storage disease type ia (GSDIa), Sci. Rep. 7 (2017) 44408.

[27] L. Hannibal, J. Theimer, V. Wiringet, K. Klotz, I. Bierschenk, R. Nitschke, U. Spiekerkoetter, S.C. Grunert, Metabolic profiling in human fibroblasts enables subtype clustering in glycogen storage disease, Front. Endocrinol. (Lausanne) 11 (2020), 579981.

[28] E.D. Brooks, D.J. Landau, J.I. Everitt, T.T. Brown, K.M. Grady, L. Waskowicz, C.R. Bass, J. D'Angelo, Y.G. Afdaw, K. Williams, P.S. Kishnani, D.D. Koeberl, Long-term complications of glycogen storage disease type ia in the canine model treated with gene replacement therapy, J. Inherit. Metab. Dis. 41 (2018) 965–976.

[29] C.P. Sentner, I.J. Hoogeveen, D.A. Weinstein, R. Santer, E. Murphy, P. J. McKiernan, U. Steuerwald, N.J. Beauchamp, J. Taybert, P. Laforet, F.M. Petit, A. Hubert, P. Labruine, G.P.A. Smit, T.G.J. Derks, Glycogen storage disease type III: diagnosis, genotype, management, clinical course and outcome, J. Inherit. Metab. Dis. 39 (2016) 697–704.

[30] P. Forny, P. Burda, P. Bode, M. Rohrbach, Is serum biotinidase enzyme activity a potential marker of perturbed glucose and lipid metabolism? JIMD Rep. 57 (2021) 58–66.