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Case Report

Clinical, biochemical and mutational findings in biotinidase deficiency among Malaysian population

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ABSTRACT

Introduction: Biotinidase deficiency (BD) is an autosomal recessively inherited disorder characterized by developmental delay, seizures, hypotonia, ataxia, skin rash/eczema, alopecia, conjunctivitis/visual problem/optic atrophy and metabolic acidosis. Delayed diagnosis may lead to irreversible neurological damage.

Methodology: Clinically suspected patients were screened for biotinidase level by a fluorometry method. Profound BD patients were confirmed by mutation analysis of *BTD* gene.

Results: 9 patients had biotinidase activity of less than 77 U. 3 patients (33%) had profound BD while 6 patients (67%) had partial BD. Compound heterozygous mutations were detected at c.98_104delinsTCC p.(Cys33Phefs*36) in Exon 2 and c.833T > C p.(Leu278Pro) in Exon 4 in two patients and a homozygous mutation at c.98_104delinsTCC p.(Cys33Phefs*36) in Exon 2 in another patient.

Conclusion: Correct diagnosis lead to early treatment and accurate management of patient. Biochemical screening of BD in symptomatic child is prerequisite to determine enzyme status however molecular confirmation is vital in differentiating individuals with profound biotinidase deficiency from partial biotinidase deficiency and also individuals' carriers.

1. Introduction

Biotin, a water-soluble B complex vitamin, is the coenzyme for four biotin-dependent carboxylases in humans essential for gluconeogenesis, fatty acid synthesis and the catabolism of several branched-chain amino acids [1,2]. Biotinidase deficiency (BD); OMIM: 253260 is an autosomal recessive inherited disorder in which the biotinidase enzyme is defective, hence the biotin is not recycled [3].

The clinical manifestation of BD include neurological and cutaneous features such as seizures, hypotonia, skin rash, alopecia, developmental delay, conjunctivitis, visual problems such as optic atrophy, hearing loss and metabolic acidosis [4]. BD can be categorized as profound BD (10% of mean normal activity in serum) and partial BTD (10%–30% of normal activity). The disorder can also be classified into early and late onset. Profound BD is an important condition where if untreated can result in severe metabolic compromised leading to coma or even death [2,5]. In partial BD, symptoms are milder and usually exaggerated by stress (ie: prolonged infection or fasting) [6]. Symptoms of BD can be successfully improved with pharmacological doses of oral biotin. However once vision, hearing problems and developmental delay occur, they are usually irreversible [1]. Individuals with BD treated since birth appeared to exhibit normal physical and cognitive development [7].

Biochemically, untreated individuals may exhibit metabolic ketoacidosis with or without hyperammonemia [8]. Urine organic acid profiles may show elevated excretion of 3-hydroxyisovaleric acid, lactic acid, 3-hydroxypropionic acids and 3-methylcrotonylglycine as well as elevation of 3-hydroxyisovalerylcarnitine (C5-OH) from dried blood spot or plasma acylcarnitine analysis [9]. Organic acid findings can be variable and affected children do not always exhibit ketoacidosis or abnormal metabolites in the urine [10]. Hence, biochemical diagnosis of BD is based on demonstration of deficient enzyme activity in whole

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Table 1
Clinical, biochemical, mutational findings of patients with profound and partial biotinidase deficiency. The mean activity of biotinidase from the local population was 250 U (equivalent to 250 nmol/min/dL of blood) with standard deviation of 122 U. The cut- off for profound biotinidase deficiency (25 U) was derived from 10% from the mean and the cut-off for partial biotinidase deficiency (77 U) was 30% from the mean.

Bil	Name	Diagnosis	Signs/symptoms	Biotinidase level (U)	Amino acids/acylcarnitine profile	Urine organic acid profile	BTD gene analysis
1	Patient 1	Partial BD	Fever, metabolic acidosis, eczema rash, sepsis	46	Normal profile	Normal profile	N/A
2	Patient 2	Profound BD	Seizure, hair loss, eczema, recurrent skin infections	11.4	Mild elevation of valine. Other AA/AC profiles are normal.	Normal profile	Compound heterozygous mutations c.98_104delinsTCC (p. Cys33Phefs*36) in Exon 2 and c.833 T > C p. (Leu278Pro) in Exon 4
3	Patient 3	Profound BD	Eczema, recurrent skin infection, seizures	12.2	Mild elevation of valine and arginine. Other AA/AC profiles are normal.	Normal profile	
4	Patient 4	Partial BD	Poor feeding. Brought in dead at Day 3 of life. Postmortem showed cardiomegaly.	73	Moderate elevation of C5OH with increase C5OH:C3 ratio.	No sample for urine organic acid analysis	No mutation for BTD gene Insufficient sample for HLCS gene
5	Patient 5	Partial BD	Neonatal jaundice, seizures	66	Normal profile	Mild excretion of 2-ketoglutarate and fumarate. No other significant peak noted	N/A
6	Patient 6	Profound BD	Encephalopathy, shocked, developmental delay, alopecia, sparse eyebrows, truncal hypotonia, brisk reflexes, bilateral optic atrophy, bilateral mixed hearing loss	17	Marked elevation of C5OH, markedly increased C5OH:C3,C5OH:C8 and C5OH:C3 ratios.	Profile showed large peak of 3-OH isovalerate, moderate peak of 3-OH propionate and small peak of 3-methyl crotonylglycine, small peak of lactic acidosis and ketones	Homozygous mutation c.98_104delinsTCC p. (Cys33Phefs*36) in Exon 2
7	Patient 7	Partial BD	Worsening skin rashes, poor weight gain	63	N/A	N/A	No mutation for BTD gene
8	Patient 8	Partial BD	Normal baby	56	Normal profile	Not requested in view of no acute symptoms	One heterozygous mutation at c.579_581del p. (Gln193_Phe194delinsHis) in Exon 4
9	Patient 9	Partial BD	Late premature baby, seizures, poor feeding, respiratory distress, hypoglycemia metabolic acidosis, hyperammonemia, encephalopathy	37	Marked increase in C3 acylcarnitine and C3:C2 ratio. Normal C5OH.	Large peak of 3-hydroxybutyrate, 3-hydroxyisovalerate and large peak of 3-hydroxypropionate. However, no 3-methylcrotonylglycine peak was detected.	Compound heterozygous mutations c.968A > G p.(His323Arg) and c.1130G > A p.(Asp444His) in Exon 4

blood. Confirmation of diagnosis is by molecular genetic testing in which sequence analysis of BTD is performed first and if there is only one or no pathogenic variant found, then followed by gene-targeted deletion/duplication analysis [11].

BTD gene has been characterized and is located on chromosome 3p25 which consists of four exons. *BTD* gene spans about 1629 bp encoding for 543 amino acids [12]. Currently, there are about 252 mutations in *BTD* gene reported by the Human Gene Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk) [13]. Mutations found are random and scattered throughout the coding exons of *BTD* gene with majority being reported are missense mutation. However, some mutations have been reported to be a hot-spot in profound BTD deficiency in the United States (G98:d7i3, R538C and Q456H) [14] and one mutation has been identified as the most prevalent mutation for partial BD deficiency (D444H) [15].

In Malaysia, newborn screening for BD is not compulsory. High risk screening for BD was conducted in Institute for Medical Research (IMR) Kuala Lumpur since 2006 using fluorometry method. There were nine positive cases of BD among Malaysian population from 2006 until 2017. Three out of nine cases were classified as profound BD. Based on results of worldwide screening of BD, the incidence of the disorder is about 1 in 137,000 for profound BD and 1 in 110,000 for partial BD; giving an overall incidence of 1 in 61,000 [16].

The aim of this study is to report clinical, biochemical and mutational findings of profound and partial BD patients among Malaysian population and to discuss the outcome of affected patients. The result of this study will be helpful in establishing the definitive diagnosis of BD deficiency at the gene level, in order to provide appropriate genetic counselling.

2. Methodology

2.1. Biochemical study

All patients diagnosed with BD throughout Malaysia from 2006 until 2017 were included in this study. Clinically suspected patients were screened for biotinidase activity in the dried blood spots. Biotinidase activity was determined by fluorometry method using Neonatal Biotinidase Kit and measured by a fluorometer. Biotinyl-6-aminoquinoline (BAQ) was used as artificial substrate. The fluorescent product (6-AQ) is formed during the enzyme reaction. Clinical findings and relevant laboratory data were taken retrospectively from request forms, Laboratory Information System (LIS) and phone interviews to respective physicians. Dried blood spots (DBS) of patients with profound and partial BD were sent for DNA extraction and mutational analysis of BTD gene for diagnostic confirmation.

2.2. Molecular study

Genomic DNA was extracted from DBS using standard protocol in QIAmp DNA Blood Mini kit (Qiagen, GmbH). DNA concentrations and quality were determined using a Nanodrop (ND-100) spectrophotometer (Thermo Fisher Scientific). DNA was subjected to PCR with M13-labelled primers specific for all four exons of *BTD* gene, including exon-intron boundaries. The PCR products were purified using the QIAQuick (Qiagen, GmbH) according to manufacturer's instructions. Cycle sequencing was performed using the BigDye Terminator cycle sequencing v3.1 chemistry (Applied Biosystems, Foster City, CA, USA) followed by purification using DyeEx 2.0 Spin Kit (Qiagen, GmbH) before loading to genetic analyser 3500 ABI (Applied Biosystems, USA) for DNA sequencing. Raw data were analysed using SeqScape software to identify any DNA variants. Any identified variants were then annotated against publicly available database such as the HGMD. Pathogenicity of detected variant was evaluated using a prediction MutationTaster software (http://www.mutationtaster.org/).

3. Results

3.1. Clinical and biochemical findings

From the total of 1434 screened patients, 9 patients (0.63%) had biotinidase activity of less than 77 U (30% of mean activity). Out of that, 3 patients (33%) were diagnosed with profound BD while 6 patients (67%) had partial BD. Three patients had biotinidase activity of less than 23 U (10% from the mean activity). Diagnosis of the affected patients was confirmed by molecular analysis of *BTD* gene. Clinical findings, biochemical profiles and BTD gene analysis of positive patients was summarized in Table 1.

Patient 1 presented at one year old with fever and eczema rash. She was also noted to have sepsis with metabolic acidosis. Otherwise, there were no other significant findings. Biotinidase was 46 U. Amino acid and acylcarnitine profiles and urine organic acid profiles were otherwise normal. She was not treated with any oral biotin however, was lost to follow up.

Patient 2 presented at the age of five years old with seizure, hair loss, eczema in the flexor area and recurrent episodes of skin infections at two months old. The development is normal up to nine years old. There was no family history of consanguinity. Amino acid and acylcarnitine profiles showed only mild elevation of valine with normal propionylcarnitine (C3) and 3-hydroxyisovalerylcarnitine (C5OH). Urine organic acid profile was normal. Biotinidase screening showed low level of biotinidase activity, 11.4 U. Oral biotin (10 mg BD) was started once diagnosis of BD was established. Symptoms gradually improved after the treatment and she had normal development, attended normal class in normal school with no behavioral issues.

Patient 3 is a younger brother of Patient 2 who presented at the age of eight years old with infantile onset of seizures, eczema and recurrent episodes of skin infection. Amino acid and acylcarnitine profiles showed mild elevation of valine and arginine with normal C3 and C5OH. Urine organic acid profile was normal. Biotinidase screening showed low level of biotinidase activity, 12.2 U. Oral biotin (10 mg BD) was started immediately after the diagnosis of BD. Post treatment, seizures were well controlled with occasional breakthrough brief seizures. However, the skin infections did not improve. Hence, the biotin dosage was increased to 15 mg BD. Outcome of this patient was unknown as patient was lost to follow up.

Patient 4 presented with history of poor feeding since day 2 of life and was brought in dead at day 3 of life. Antenatal history was uneventful and there was no family history of consanguinity, family history of congenital heart disease or metabolic diseases. Postmortem finding showed cardiomegaly without any other significant findings. Biotinidase activity was 73 U. Amino acids and acylcarnitine profiles showed moderate elevation of C5OH with increased C5OH:C3 ratio. There was no urine sample available for urine organic acid profiles. BTD gene mutation study did not detect a mutation.

Patient 5 presented at day 14 of life with neonatal jaundice not requiring exchange transfusion. During admission, she developed 2 episodes of seizure and was treated as presumptive meningitis. Ultrasound of the cranium was normal. Amino acids and acylcarnitine profiles showed normal profile while urine organic acid profiles showed mild excretion of 2-ketoglutarate and fumarate with no other significant peaks. Biotinidase screening showed biotinidase activity to be 66 U. She had another admission for complex febrile seizure with scalp abscess the following year. However, no further metabolic work up was done during this admission. She subsequently defaulted her clinic visits.

Patient 6 is the youngest of three siblings from a non-consanguineous marriage. There was no family history of early death or chronic neurological disorders. She was admitted to the intensive care at the age of five months when she presented with encephalopathy and in a shocked state. She was managed then with respiratory support, inotropes, anticonvulsants and empirical antibiotics. Patient subsequently recovered but had severe neurological deficit. Investigation showed negative septic screening. However, there were significant lactic ketoacidosis with increased anion gap. The diagnosis of biotinidase deficiency was suspected clinically due to history of developmental delay, alopecia and sparse eyebrows. Acylcarnitine profile revealed marked elevation of C5OH, markedly increased C5OH:C3, C5OH:C8 and C5OH/:C0 ratios while urine organic acid profiles showed large peak of 3-OH isovalerate, moderate peak of 3-OH propionate and small peak of 3-methyl crotonylglycine with small peak of lactate and ketones. Subsequent biotinidase screening showed significantly low biotinidase activity of 1 U. Following commencement of oral biotin 10 mg OD, patient showed improvement in the hair growth, however neurological deficit was irreversible. Despite good compliance to biotin therapy, she was noticed to have marked global developmental delay, truncal hypotonia with hypertonic limbs and brisk reflexes, bilateral optic atrophy and bilateral mixed hearing loss on her clinic follow up at the age of four years old. She is currently wheelchair bound, on hearing aid and is completely dependent for her daily activity living.

Patient 7 had underlying history of thalassemia trait, presented with worsening of skin rashes for 3 months and poor weight gain for the past 4 months. He was investigated for biotinidase deficiency in view of the persistent skin rashes and the biotinidase screening showed partial low activity of biotinidase (63 U). Amino acids and acylcarnitine or urine organic acid screening were not done for this patient.

Patient 8 was otherwise a normal baby who had a newborn screening for IEM as part of the screening policy in the private sector. The amino acids and acylcarnitine profiles were normal. However, the biotinidase screening showed borderline low level of biotinidase activity (56 U).

Patient 9 was a late premature baby at 36 weeks born via spontaneous vaginal delivery with birth weight of 2.03 kg. He had good APGAR score. He initially presented on day three of life with history of seizures, poor feeding, respiratory distress, sepsis, hypoglycaemia and severe metabolic acidosis with raised anion gap. There was no history of parental consanguinity. After a bolus injection of D10% dextrose, the repeat blood glucose was 5.6 mol/L. The initial ammonia was 538 mmol/L which then quickly rised to 1614 mmol/L. He was intubated and ventilated and started empirically on IV C-Penicillin and IV Cefotaxime. Peritoneal dialysis was started as he failed to respond to sodium benzoate, sodium phenylbutyrate and arginine. Amino acids and acylcarnitine profiles showed marked increase in C3 and C3:C2 ratio without elevation of C5OH which was suggestive of either methylmalonic aciduria or propionic aciduria. The urine organic acid profiles showed large peak of 3-hydroxybutyrate, 3-hydroxyisovalerate and large peak of 3-hydroxypropionate with no 3-methylcrotonylglycine peak detected. The clinical diagnosis based on the initial metabolic results were Propionic aciduria. However, patient's condition deteriorated quickly and became encepahalopathic with worsening renal function, hyperammonemia and metabolic acidosis despite peritoneal dialysis. The clinical impression was then changed to multiple carboxylase deficiency. Blood for biotinidase enzyme was taken after patient has passed away and showed partial BD.

3.2. Mutations in BTD gene

Mutational analysis was performed for profound BTD Patient 2, 3 and 6 and revealed two heterozygous mutations in both siblings Patient 2 and 3 and one homozygous mutation in Patient 6. Mutations detected were c.98_104delinsTCC p. (Cys33Phefs*36) in Exon 2 and

c.833 T > C p. (Leu278Pro) in Exon 4. Both mutations had been reported previously in Human Gene Mutation Database (HGMD). Mutation p. (Cys33Phefs*36) was reported by Pomponio (1995) while mutation p. (Leu278Pro) was described in another study by Pomponio (1997). MutationTaster software predicted both mutations as disease causing.

BTD gene analysis was also performed in 4 out of 6 partial biotinidase deficiency patients. Patient 8 and 9 exhibited mutation/s and none was detected in Patient 4 and 7. We found only one heterozygous in-frame deletion in Patient 8 detected at c.579_581del p. (Gln193_Phe194delinsHis) in Exon 4. This variant is a reported polymorphism in dbSNP (ncbi.nlm.nih.gov/snp/) even though it has not been present in 1000 Genomes Project and ExAC databases. Whereas in Patient 9, a compound heterozygous mutation was detected at c.968A > G p.(His323Arg) and c.1130G > A p.(Asp444His), both located in Exon 4. The p.(Asp444His) and p.(His323Arg) were reported in HGMD for partial BD. Both mutations were present in 1000 Genomes Project and ExAC databases, suggesting polymorphism. Molecular analysis for Patient 1 and 5 were not done as patient were lost to follow up and parents were uncontactable.

4. Discussion

Clinical manifestation of BD varied but, most affected children do not exhibit symptoms immediately after birth. They commonly presented clinically at several months of age or in adolescence or adulthood [17]. Symptoms of untreated profound biotinidase deficiency usually appear between the age of 1 week and 10 years with a mean age of 3.5 months [18]. The early symptoms of profound BD often affect the nervous system and most children (over 70%) had seizures, hypotonia, skin rash or alopecia [10]. The majority of patients with partial BD showed milder manifestations as compared to those with profound BD and most of their symptoms are usually exaggerated by stress such as prolonged fasting or infection [6]. Both Patient 2 and 3 whom are siblings presented with infantile onset seizure, eczema and recurrent episodes of skin infections. Patient 6 presented with more serious presentation of encephalopathy at the time of diagnosis preceded by developmental delay with clinical findings of alopecia and sparse eyebrows. The remaining patients in our cohort presented with non-specific symptoms ranging from fever, respiratory distress, poor feeding, poor weight gain, developmental delay, sepsis and metabolic acidosis. Two patients in our cohort, patient 4 and 9 had acute early presentation within few days of life and early neonatal mortality. BD usually present later in life in contrast to holocarboxylase synthetase deficiency. Both patients 4 and 9 had different clinical presentation from typical BD patients and there were no BTD gene mutation detected in both patients eventhough biotinidase enzyme was in the range of partial biotinidase deficiency. We would have proceeded with HLCS gene mutation analysis for patient 4 and 9, unfortunately there were insufficient DNA sample for both patients. Patient 8 was a normal asymptomatic baby who was screened for biotinidase deficiency as part of newborn screening in a non-government hospital.

Biochemically, urine organic profiles in individuals with BD may show either normal or elevated 3-hydroxyisovaleric acid, 3-hydroxypropionic acid, 3-methylcrotonylglycine and methylcitric acid [10]. Although presence of abnormalities in urine organic acid and acylcarnitine analysis maybe suggestive of BD they should not be used as the sole test for diagnosis of BD as the biochemical abnormalities may be shared by other disorders such as 3-hydroxy-3-methylglutaric acidemia, 3-methyl-crotonyl-glycinuria (3-MCC) and glutaric aciduria type I [10]. In Patient 2 and 3, the urine organic acid and acylcarnitine analysis did not suggest the diagnosis of BD despite profound low biotinidase level. In Patient 1 and 8 there were borderline low level of biotinidase activity but, normal amino acid and acylcarnitine profiles with normal profile of urine organic acids. Although it cannot be proven, there are possibilities of pre analytical errors in the sample of patients due to prolonged storage of blood spot under room temperature which can reduced the enzyme activity. Long term storage of samples at room temperature, humidity or wet samples may result in significant loss of enzyme activity [10]. Patient 1 and 5 were lost to follow up and uncontactable, resulting to failure of blood collection for further molecular analysis.

BTD gene analysis revealed compound heterozygous for known mutations that caused profound BD in Patient 2 and 3, a deletion and insertion mutation at c.98_104delinsTCC p.(Cys33Phefs*36) in Exon 2 causing frameshift and leads to premature stop codon and substitution at c.833 T > C p.(Leu278Pro) in Exon 4. Both mutations are predicted to be disease causing by MutationTaster [20]. Patient 6 has biotinidase activity in the profound deficiency range. This patient had a homozygous mutation at c.98_104delinsTCC p. (Cys33Phefs*36) in Exon 2 which is also known as G98: d7i3, a mutational hotspot that seems to be a common mutation causing profound biotinidase deficiency, occuring in at least one allele of about 50% of symptomatic children [9]. This mutation results in a frameshift in the coding sequence of the putative signal peptide sequence which predicted to introduce a premature stop codon and lead to a production of a truncated protein. The resulting protein become truncated and does not simulate a normal structure and function of biotinidase. Hence the mutation resulted in a near complete absence of biotinidase protein as demonstrated in Patient 6 with a severe clinical manisfestation. The mutation found in Patient 6 was confirmed with parental samples that showed heterozygosity. It seems that deletion/insertion mutation (c. 98_104delinsTCC) is also common in our patients although the parents of affected patients had claimed no consanguinity. This observation is consistent with the data published so far [21,22].

Partial BD patients (Patient 8 and 9) had biotinidase activity in the partial deficiency range (10%–30% of mean normal activity). Patient 8 has a borderline low level and the p.(Gln193_Phe194delinsHis) mutation is the likely cause of the enzyme activity. Patient 9 had enzyme activity close to profound BD range. This might be due to the presence of two heterozygous p.(Asp444His) mutation which is the common cause of partial BD [15] and p.(His323Arg) mutation that has been reported in partial BD [15] as well. As for the rest of the suspected partial BD patients (Patient 1,4,5 and 7) with no mutations, we suggest for analysis of other contributing gene i.e. *HLCS* gene causing holocarboxylase synthetase deficiency due to overlapping symptoms in early-onset or infantile multiple or combined carboxylase deficiency [11].

Children with profound BD are treated with lifelong oral biotin supplementation. Whilst biotin treatment is effective and safe, there are some areas of uncertainty on the appropriate dose of biotin for partial BD treatment [4]. However, it has been suggested that lower doses of biotin, 5 to 10 mg/day maybe sufficient for the treatment of children with partial BD [6]. Individuals with profound BD whom identified early within the newborn period and started on biotin therapy with good compliance, may have normal physical and cognitive development [7]. Patient 2, 3 and 6 were treated at the time of diagnosis and remained compliant to biotin treatment. However, they were diagnosed late and beyond neonatal period as there is no policy on newborn screening for BD in the country. Early detection will prevent the irreversible neurological complication in these patients. As potential serious consequences may arise, it is also important to identify children with partial BD [6].

Post-treatment, the outcomes were good for Patient 2 whereby only occasional breakthrough of seizure was seen in Patient 3. In contrary, Patient 6 had severe neurological deficits. The very low level of enzyme caused very small concentration of biotin for the nervous system to functioning well which led to severe brain damage. Based on a 20 years old follow up study, oral biotin alleviates most of clinical symptoms of BD in symptomatic patients, but does not reverse neither optic nerve atrophy nor hearing loss [23]. However, introduction of biotin treatment during presymptomatic stage of the disease had shown to prevent the occurrence of symptoms including optic atrophy [23]. The

outcomes were not assessed among patients with partial BD since they were not treated with oral biotin and most were lost to follow up.

Management of patients with BD should be in a holistic manner which includes yearly ophthalmologic examination, auditory testing, and assessment by a metabolic specialist. Testing of biotinidase activity is recommended in asymptomatic and symptomatic siblings of affected individuals [11]. In our population, the newborn screening for biotinidase is not mandatory and high risk screening was carried out for BD. Newborn screening of BD should be considered as it is safe, inexpensive and the condition is easily treatable. Early diagnosis had also shown to improve prognosis and outcome in patients with BD. Biochemical screening of BD with fluorometry method in symptomatic child is prerequisite to determine enzyme status before the molecular confirmation. Patients with unexplained and intractable neurological and cutaneous manifestations should be investigated for BD [19]. It is highly recommended that patients with absent/low biotinidase activity to be further confirmed by mutation analysis to ensure early and accurate treatment to prevent irreversible complications. Mutation analysis for biotinidase deficiency is readily available and useful for confirmatory testing to help differentiate between individuals with profound and partial biotinidase deficiency and also individuals' carriers.

Declaration of Competing Interest

The authors declare no conflict of interest.

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