



High frequency of biotinidase deficiency in Italian population identified by newborn screening

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ABSTRACT

The biotinidase (BTD) enzyme is essential for recycling biotin, a water-soluble B-complex vitamin that is the coenzyme of four carboxylases involved in fatty acid synthesis, amino acid catabolism and gluconeogenesis. If untreated, total or partial BTD deficiencies lead to an autosomal recessive inherited organic aciduria whose clinical features, mainly presenting in the first years of life, include, seizures, skin rash, and alopecia. Based on residual BTD enzyme activity it is possible to identify partial or total biotinidase deficiency. The incidence of profound and partial biotinidase deficiency worldwide is estimated to be about 1 in 60,000.

We report twelve years of experience in the newborn screening of biotinidase deficiency on 466,182 neonates. When a positive screening result occurred, a clinical evaluation was made of the patient and genetic counselling was offered to the family. Molecular analysis the BTD gene was carried out in all recalled neonates.

Newborn screening lead to the identification of 75 BTD deficiencies with an incidence of about 1:6,300 births, ten times higher than the reported worldwide incidence. BTD deficiency was confirmed at a genomic level in all patients, demonstrating a high frequency of the p.(Asp444His) amino acid substitution and the complex allele p.(Ala171Thr)/p.(Asp444His) in the analyzed Italian newborns. Four new mutations (two small deletions, one stop mutation and one missense mutation) and a new combined allelic alteration were identified.

Our data suggests that there is a high incidence of the biotinidase defect in the Italian population, most likely due to the high frequency of certain mutations.

1. Introduction

Biotinidase (BTD) deficiency (OMIM 253260) is an autosomal recessive inherited metabolic disorder [1]. The biotinidase enzyme is essential for recycling biotin, a water-soluble B-complex vitamin that is the coenzyme of four carboxylases involved in fatty acid synthesis, amino acid catabolism and gluconeogenesis [1]. Two distinct phenotypes have been identified based on residual enzyme activity: profound biotinidase deficiency if biotinidase activity is less than 10% of normal

enzymatic activity, and partial biotinidase deficiency if enzymatic activity is 10–30% of normal enzymatic activity [1].

Patients with untreated BTD deficiency typically develop seizures, skin rash, and alopecia with accompanying acidosis and a characteristic organic acidemia due to multiple carboxylase deficiency in the first few years of life [1,2]. Many children have ataxia, developmental delay, conjunctivitis, hearing loss, and visual problems, including optic atrophy. Some affected individuals have severe metabolic impairment that results in coma or death [1]. Early detection and the prompt

Abbreviations: BTD, Biotinidase; HGMD, Human Gene Mutation Database; DBS, Dried blood spot; DNA, DeoxyriboNucleic Acid; PCR, Polymerase Chain Reaction; C5-OH, 3-OH-isovalerylcarnitine; LC-MS/MS, Liquid Chromatography Tandem Mass Spectrometry; GC-MS, Gas chromatography–mass spectrometry; IQ, Intelligence Quotient; WISC, Wechsler Intelligence Scale for Children.

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introduction of oral biotin supplementation therapy prevents the neurological sequelae and clinical events [3,4]. For this reason, biotinidase deficiency meets the criteria for inclusion in newborn screening programs [5]. Many countries currently perform newborn screening for biotinidase deficiency based on determination of biotinidase activity on dried blood spots [6].

Diagnosis is confirmed by assaying biotinidase activity in serum [4]. Profound and partial biotinidase deficiencies currently have an estimated worldwide incidence of about 1 in 60,000 [4,7]. The biotinidase gene has been mapped to chromosome 3p25, and the genomic structure of the human biotinidase gene has been determined [8]. To date, 266 genetic mutations have been reported (Human Gene Mutation Professional Database HGMD <https://portal.biobase-international.com>), of which 243 mutations are correlated with a profound enzymatic defect, 23 with a partial enzymatic defect and 3 are reported in patients affected by multiple carboxylase deficiency.

A high frequency of common mutations correlated with partial or total deficiency has been described in the *BTD* gene both in patients diagnosed after developing clinical symptoms and in those identified by newborn screening [4].

We report our decade-long experience in newborn screening (approximately 39,000 neonates/year) for biotinidase deficiency in the regions of Umbria and Tuscany in Italy. Biotinidase deficiency was confirmed at a molecular level in all patients identified through newborn screening; the relatives of the proband were also studied.

2. Methods

2.1. Sample collection

Dried blood spot (DBS) samples were collected from all neonates born in Tuscany and Umbria.

Blood collection for newborn screening was performed between the first 48 and 72 h of life. Blood samples were obtained by heel stick, spotted on filter paper (903, Whatmann), dried and sent by courier to the screening centre as previously reported [9]. Special protocols for premature infants, newborns receiving parenteral nutrition and transfused newborns were applied. [9].

2.2. Biotinidase determination in dried spot and serum

Biotinidase activity can be determined by the development of colour due to the release of *p*-aminobenzoate from *N*-biotinyl-*p*-aminobenzoate, a biocytin analogue as described by Heard et al. [6]. An artificial positive control was added to each screening session; this control was created from a pool of normal blood collected in EDTA. After centrifugation, plasma was replaced V:V with 0.9% saline solution. 20 μ L of the artificial blood was spotted on filter paper and used to control the sensitivity of the screening process. Diagnosis was confirmed by quantitative colorimetric assay of serum biotinidase activity [5,10].

2.3. Mutation analysis

After clinical and genetic counselling, genomic DNA from peripheral blood was isolated in all neonates resulting positive in biochemical screening assays. PCR amplification and standard DNA sequencing of the *BTD* gene were performed as previously reported [11,12]. The GenBank reference sequence is NM_000060.3. All biological samples were obtained for storage and analysis only after family member's written informed consent had been obtained, using a form approved by the local Ethics Committee (Meyer Children Hospital, Florence, Italy).

2.4. In silico analysis

The 1000 Genomes project database (<http://www.1000genomes.org/>), including all human genetic variations from the dbSNP short

genetic variations database (<https://www.ncbi.nlm.nih.gov/snp/>) and the ExAC Browser of the Exome Aggregation Consortium, which provides a data set of over 60,000 unrelated individuals (<http://exac.broadinstitute.org>), and gnomAD browser which provides a data set spanning over 130,000 unrelated individuals (<https://gnomad.broadinstitute.org/>) were used to evaluate the polymorphic status of the identified genetic alterations. The single amino acid substitutions were analyzed by the SIFT (http://sift.jcvi.org/www/SIFT_aligned_seqs_submit.html), PolyPhen (<http://genetics.bwh.harvard.edu/pph/>), Mutation T@sting (<http://www.mutationtaster.org/>) and Align GVGD (<http://agvgd.hci.utah.edu/>) software packages.

3. Results

Official legislation (Region Law n.800 of 3/8/2004) introduced newborn screening for about 40 metabolic diseases, by amino acids and acylcarnitine analysis, in Tuscany in November 2004 and in Umbria in January 2010. As of December 2019, 579,812 newborns had been screened (approximately 39,000/year) through an expanded newborn screening program. Newborn screening for biotinidase deficiency began in January 2007, and up to now 466,182 newborns have been screened.

When a positive screening result occurs, a second dried blood spot is requested to re-evaluate the biotinidase activity. The average recall rate over ten years was 0.2%. This higher than expected recall rate must be attributed to the incorrect collection of the samples (poor impregnation of blood on filter paper, high humidity, failure to allow the filter paper to dry sufficiently before packing in plastic bags or heat exposure) and to enzymatic immaturity [4].

About 10% of the recalled newborns had a confirmed positive result on retesting.

Biotinidase activity in serum was measured in all newborns with positive retesting. In addition, plasma acylcarnitines in LC-MS/MS and urinary organic acid profiles in GC-MS were performed to check for characteristic abnormalities found in patients with biotinidase deficiency such as elevated excretion of 3-hydroxyisovaleric, lactic, and 3-hydroxypropionic acids and 3-methylcrotonylglycine in urine as well as mildly elevated 3-OH-isovalerylcarnitine (C5-OH) in plasma.

Serum biotinidase assay revealed an enzymatic activity of less than 30% in 75 babies (10 profound and 65 partial defects, Table 1).

After clinical evaluation and genetic counselling, molecular analysis of the *BTD* gene carried out in all recalled neonates with a decrease in biotinidase activity confirmed the genetic defect in all of them (Table 1). All parents were screened to confirm their heterozygous carrier status.

Metabolic clinical management, genetic counselling and follow-up of all diagnosed patients were provided by our metabolic unit. Until two years ago all patients were put on biotin therapy (partial *BTD* patients with 5 mg/die and profound *BTD* patients with 5–20 mg/ die). After a careful clinical and biochemical evaluation based on many years of experience and careful review of the data reported in the literature [3], we decided to stop treating patients homozygous for the D444H mutation and to recall them for clinical and biochemical follow up once a year. However, parents and primary care doctors are alerted to report any possible emerging symptoms, such as frequent rashes or alopecia, that can be ascribed to biotin deficiency [13]. To date all *BTD* patients are well without clinical symptoms.

The p.(Asp444His) amino acid substitution was identified in 64 patients with partial biotinidase deficiency; only in one case was the partial defect due to a p.(Arg148His) amino acid substitution. As previously reported [14], these data confirm that partial biotinidase deficiency is usually due to the presence of a p.(Asp444His) amino acid substitution in homozygote status or in a combination in one allele with a genetic mutation that results in profound biotinidase deficiency in the other allele (Table 1).

The p.(Asp444His) amino acid substitution was also found in cis with p.(Ala171Thr) as a combined allelic alteration. This complex allele p.(Ala171Thr)/p.(Asp444His) correlates with profound *BTD* deficiency

Table 1

Biotinidase activity and mutation analysis results of newborns with BTDA enzymatic activity <30%. The new mutations identified in BTDA patients are in bold.

| Genotype | Number of patients per genotype | Range of serum Biotinidase activity (nmol/min/mL) (n.v. 6.11–20.5; mean 13.8) | ALLELE 1 | ALLELE 2 | DEFICIT |
|----------|---------------------------------|---|---------------------------|----------------------------------|-----------------|
| 01 | 1 | 3.3 | p Arg148His | Thr351Lysfs*12 | PARTIAL |
| 02 | 10 | 2.3–3.8 | p.Asp444His | p.Asp444His | PARTIAL |
| 03 | 2 | 2.5–3.7 | p.Asp444His | p.Arg79Cys | PARTIAL |
| 04 | 1 | 2.1 | p.Asp444His | p.Arg211Cys | PARTIAL |
| 05 | 1 | 2.2 | p.Asp444His | p.Ala439Asp | PARTIAL |
| 06 | 1 | 2.4 | p.Asp444His | p.Asp444Tyr | PARTIAL |
| 07 | 1 | 1.8 | p.Asp444His | p.His447Tyr | PARTIAL |
| 08 | 1 | 3 | p.Asp444His | p.Tyr454Cys | PARTIAL |
| 09 | 12 | 1.4–3.2 | p.Asp444His | p.Gln456His | PARTIAL |
| 10 | 1 | 2.2 | p.Asp444His | p.Trp487* | PARTIAL |
| 11 | 1 | 1.8 | p.Asp444His | p.Thr492Ile | PARTIAL |
| 12 | 5 | 2.7 | p.Asp444His | p.Pro497Ser | PARTIAL |
| 13 | 1 | 1 | p.Asp444His | p.Trp514* | PARTIAL |
| 14 | 2 | 3.4–1.7 | p.Asp444His | p.Thr532Met | PARTIAL |
| 15 | 1 | 1.4 | p.Asp444His | c.203_206dupTCCT | PARTIAL |
| 16 | 1 | 2.3 | p.Asp444His | c.386dupT | PARTIAL |
| 17 | 3 | 2.3–2.8 | p.Asp444His | c.98_104delGCGGCTGinsTCC | PARTIAL |
| 18 | 1 | 2 | p.Asp444His | c.933delT | PARTIAL |
| 19 | 2 | 2.4–2.5 | p.Asp444His | c.1052delC | PARTIAL |
| 20 | 1 | 2.9 | p.Asp444His | p.Asp444His + p.Arg157His | PARTIAL |
| 21 | 16 | 1.6–3.4 | p.Asp444His | p.Ala171Thr + p.Asp444His | PARTIAL |
| 22 | 3 | 0.4–0.9 | p.Ala171Thr + p.Asp444His | p.Ala171Thr + p.Asp444His | PROFOUND |
| 23 | 1 | 0.5 | p.Gln456His | p.Ala171Thr + p.Asp444His | PROFOUND |
| 24 | 2 | 0.6–0.7 | p.Thr532Met | p.Thr532Met | PROFOUND |
| 25 | 2 | 0.1 | p.Gln456His | p.Gln456His | PROFOUND |
| 26 | 1 | 0.1 | p.Tyr93Cys | p.Gln456His | PROFOUND |
| 27 | 1 | 0.05 | p.Phe232Cys | p.Phe232Cys | PROFOUND |

[15] and was found in 21 patients, three of whom had inherited it at a homozygous level.

In patient with genotype 20 (Table 1) the p.(Asp444His) amino acid substitution was in cis with p.(Arg157His) causing a new combined severe allelic alteration. The p.(Arg157His) mutation was first reported by Pomponio et al. [16] as a cause of profound BTDA deficiency. The combined allele was confirmed in the patient's father at a molecular level whose BTDA enzymatic activity was 37%.

Our studies also show a high frequency (Table 1) of the p.(Gln456His) amino acid substitution proving that this mutation is the

most common cause of profound biotinidase deficiency in children diagnosed by newborn screening as previously described [17].

Four new mutations: c.386dupT p.(Leu129FfsX23); c.203_206dup p.(Ser70Profs*23)]; p.(Trp487*) and the p.(Asp444Tyr) were also found in our patients (Table 1).

The newly identified deletions [c.386dupT p.(Leu129FfsX23); c.203_206dup p.(Ser70Profs*23)] and the stop mutation p.(Trp487*), giving rise to frame shifts in the BTDA coding region, are severe mutations. In silico predictions and the residual BTDA activity were considered in order to make a genotype/phenotype correlation for the new p.

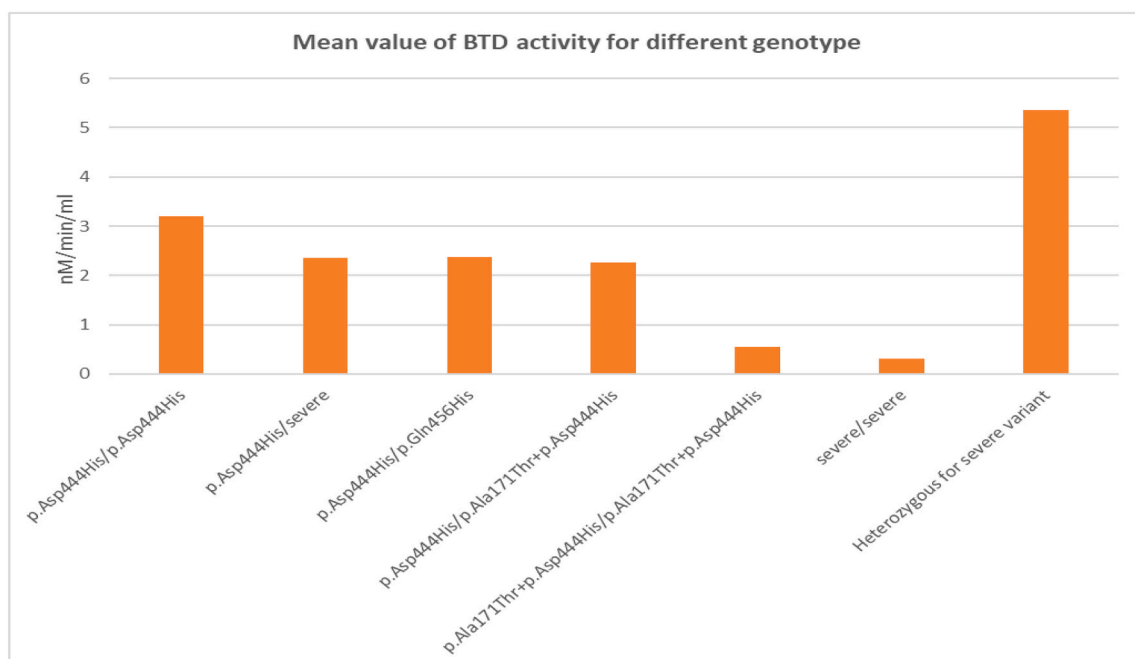


Fig. 1. Mean values of enzyme assays for different genotype groups.

(Asp444Tyr) missense mutation. The new p.(Asp444Tyr) mutation was detected in a patient who was compound heterozygous with the mild p.(Asp444His) mutation, which gave rise to a partial BTD defect in the corresponding blood sample. In silico analysis showed that this amino acid change probably damages the resulting BTD protein.

In the 1000 Genomes, EXAC browser and GnomAD browser all the new variants identified were present at very low frequencies (ranging from 0.0003 to 0.003).

The enzymatic activities of the patients' genotypes and of the carriers are shown in Fig. 1.

4. Discussion

We report our experience in newborn screening for biotinidase deficiency in about 500,000 neonates. We identified 75 BTD deficiencies with an overall incidence of about 1:6.300 births, ten times higher than the worldwide incidence [4,7]. The difference between the incidence observed in retrospective clinical studies and our newborn screening study suggests that the disorder may be under-diagnosed.

Biotinidase screening based on the assay of enzymatic activity on dried blood spots, generates a high number of false positives due to prematurity, liver dysfunction and mishandling of samples. Biotinidase activity increases during the first weeks of life, and full-term newborns only have 50–70% of normal mean adult biotinidase activity [4]. In the case of a positive result, the weeks of gestation are checked to determine whether the result could be a false positive caused by enzyme immaturity. If required, a new blood spot is taken on the 14th day of life.

All patients with profound or partial biotinidase deficiency identified by colorimetric analysis during the Tuscany and Umbria newborn screening program showed normal screening results in LC-MS/MS, confirming that 3-OH-isovalerylcanitine (C5-OH) is not a marker for biotinidase deficiency.

In addition, the plasma acylcarnitines and urinary organic acids performed at the time of the recall did not reveal any alterations, confirming that biochemical alterations are present in undiagnosed BTD patients who are already symptomatic or in BTD patients on therapy who need to increase their dose of biotin.

Quantitative assay of biotinidase activity in serum can confirm the diagnosis of biotinidase deficiency. However, the enzymatic assay may not establish whether the child has a partial deficiency or is heterozygous for a profound deficiency [4] and so mutation analysis is needed. The classification is important in determining whether therapeutic management with oral biotin supplementation is appropriate [3,7].

This study reports on 10 patients affected by profound biotinidase deficiency and 65 by partial deficiency (Table 1). The identification in 68 patients (88/150 alleles) of the c.1330G > C genetic variant leading to the p.(Asp444His) amino acid substitution (by itself or as a complex allele) suggests that this genetic variant is the most common in the population of central Italy (Tuscany and Umbria). The p.(Ala171Thr) mutation in combination with the p.(Asp444His) mutation is a known double mutation that causes profound biotinidase deficiency in the allele and a 52% decrease in activity of the aberrant enzyme [10]. Twenty patients carried the complex allele p.(Ala171Thr)/p.(Asp444His), three of them in homozygote with allele frequencies of 16%, in agreement with the literature [18].

Molecular studies identified 23 different pathogenic variants, including 4 new genetic mutations and a new combined allelic alteration. The new p.(Asp444His)/p.(Arg157His) complex allele causes low biotinidase activity due to the combined effect of mutations. The parent carrying the complex allele had an enzymatic activity of 37%.

The variants p.(Gln456His) and p.(Thr532Met) were the most common variants in our patients with allele frequencies of 12% and 4% respectively.

Inheritance was confirmed by genetic analysis of the parents. Family studies led to the identification of partial biotinidase deficiency in several relatives. The brother of the patient with genotype 18 was

dyslexic and had difficulty at school. His IQ, as tested by the Wechsler Intelligence Scale for Children (WISC), was below-average. It is possible that this clinical picture may be related to a biotinidase deficiency that was not detected early and treated with biotin, as was the case for the patient's relative described by Funghini et al. [11].

Some authors hypothesised that learning and cognitive difficulties arise as a result of reduction of free biotin level in the brain where the content is more important rather than in other tissues, so they recommended biotin therapy in patients with enzymatic activity lower than 15% [19].

5. Conclusions

Our extensive pre and post screening experience shows that the early introduction of biotin therapy can prevent clinical symptoms in all patients diagnosed with BTD by newborn screening.

The genotyping of patients diagnosed with biotinidase deficiency has led to a better understanding of the disease. Our studies show an incidence ten times higher than the reported worldwide incidence of the biotinidase defects in the Italian population due to the high frequency of certain mutations.

Authors contributions

All authors participated in the drafting of the manuscript. Each of the authors confirms that this manuscript has not been previously published and is not currently under consideration by any other journal. Additionally, all of the authors have approved the contents of this paper and have agreed to the Life Sciences's submission policies.

Declaration of Competing Interest

None of the authors has a financial or non-financial interests that could influence this paper.

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