Genetics inMedicine

Laboratory diagnosis of biotinidase deficiency, 2017 update: a technical standard and guideline of the American College of Medical Genetics and Genomics

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Biotinidase deficiency is an autosomal recessively inherited disorder of biotin recycling that is associated with neurologic and cutaneous consequences if untreated. Fortunately, the clinical features of the disorder can be ameliorated or prevented by administering pharmacological doses of the vitamin biotin. Newborn screening and confirmatory diagnosis of biotinidase deficiency encompasses both enzymatic and molecular testing approaches. These guidelines were developed to define and standardize laboratory procedures for enzymatic biotinidase testing, to delineate situations for which follow-up molecular testing is warranted, and to characterize variables that can influence test performance and interpretation of results.

Genet Med advance online publication 6 July 2017

Key Words: biotinidase deficiency; clinical genetic testing; multiple carboxylase deficiency; newborn screening; technical standards and guidelines

INTRODUCTION

Biotinidase deficiency, also known as late-onset multiple carboxylase deficiency, is an autosomal recessively inherited disorder of biotin recycling associated with secondary alterations in amino acid, carbohydrate, and fatty acid metabolism.[1,2] The disorder is caused by absent or markedly deficient activity of biotinidase, a cytosolic enzyme that liberates free biotin from biocytin during the normal proteolytic turnover of holocarboxylases and other biotinylated proteins.[3] Biotinidase may also play a role in the biotinylation of specific proteins, such as histones.[4] The diagnosis of biotinidase deficiency is based on demonstrating deficient enzyme activity in serum or plasma.[5] Patients with profound biotinidase deficiency have less than 10% of mean normal serum activity, while patients with the partial biotinidase deficiency variant have 10–30% of mean normal serum activity and are largely asymptomatic. Confirmation of biotinidase deficiency by DNA analysis, by either allele-targeted methods or full-gene sequencing, may be useful. Biotinidase testing may be part of a larger workup in a child who exhibits clinical features suggestive of the disease, but more frequently is performed on asymptomatic infants following abnormal newborn screening results. Many of the clinical issues and frequently asked questions about biotinidase deficiency have been addressed in review articles.[6,7] Because all newborn screening programs in the United States and many other countries worldwide include biotinidase deficiency in their panel of screened conditions, there is an increased need

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The Board of Directors of the American College of Medical Genetics and Genomics approved this guideline on 21 March 2017.

Submitted 3 May 2017; accepted 3 May 2017; advance online publication 6 July 2017. doi:10.1038/gim.2017.84

to define and standardize laboratory approaches for enzymatic biotinidase testing, to delineate situations for which follow-up molecular testing is warranted, and to characterize variables that can influence test performance and interpretation of results. These guidelines describe best laboratory practices for biotinidase deficiency testing including preanalytical considerations, test selection and performance, results interpretation, and appropriate follow-up testing.

BACKGROUND

Biochemical and molecular characteristics of biotinidase

Biotinidase (E.C. 3.5.1.12) is a 70–80 kDa glycoprotein that is ubiquitously expressed and detectable in multiple tissues including serum, leukocytes, fibroblasts, and liver.[8] The enzyme recycles biotin, a water-soluble cofactor necessary for the function of four carboxylases: propionyl-CoA carboxylase (E.C. 6.4.1.3), 3-methylcrotonyl-CoA carboxylase (E.C. 6.4.1.4), pyruvate carboxylase (E.C. 6.4.1.1), and acetyl-CoA carboxylase (E.C. 6.4.1.2).[9] An inherited disorder of biotin recycling or utilization, from a deficiency of biotinidase (MIM 253260) or holocarboxylase synthetase (E.C. 6.3.4.10; MIM 253270), leads to secondary disruptions in the activities of the aforementioned carboxylases that, in turn, cause impairments in the metabolic pathways of amino acids, carbohydrates, and lipids (see **Figure 1**).

Biotinidase is a monomeric enzyme encoded by a single gene (*BTD*) located on chromosome 3p25, and comprises 543 amino acid residues, including 41 amino acids of a potential signal peptide.[10,11] The gene spans at least 23 kilobases of genomic DNA and consists of four exons and three introns, reflecting a simple structural organization that facilitates gene sequencing to genotype patients.[12] Two putative translation initiation codons exist, one in exon 1 and the other in exon 2, with the second containing the N-terminal methionine of the mature enzyme.[12] The sequences upstream of exons 1 and 2 contain promoter elements consistent with the ubiquitous expression of biotinidase as a housekeeping gene. The presence of an intron between the two possible initiation codons could allow for alternative splicing, raising the possibility of tissue-specific expression of different *BTD* transcripts. In support of this idea, a consensus sequence for the liver-specific transcription factor HNF-5 is present at nucleotide position −352, although the physiologic significance of this is unknown.

Three publicly available databases of biotinidase variants are accessible online: the Leiden Open Variation Database (https://grenada.lumc.nl/LOVD2/shared1/home.php?select_db=BTD), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/),

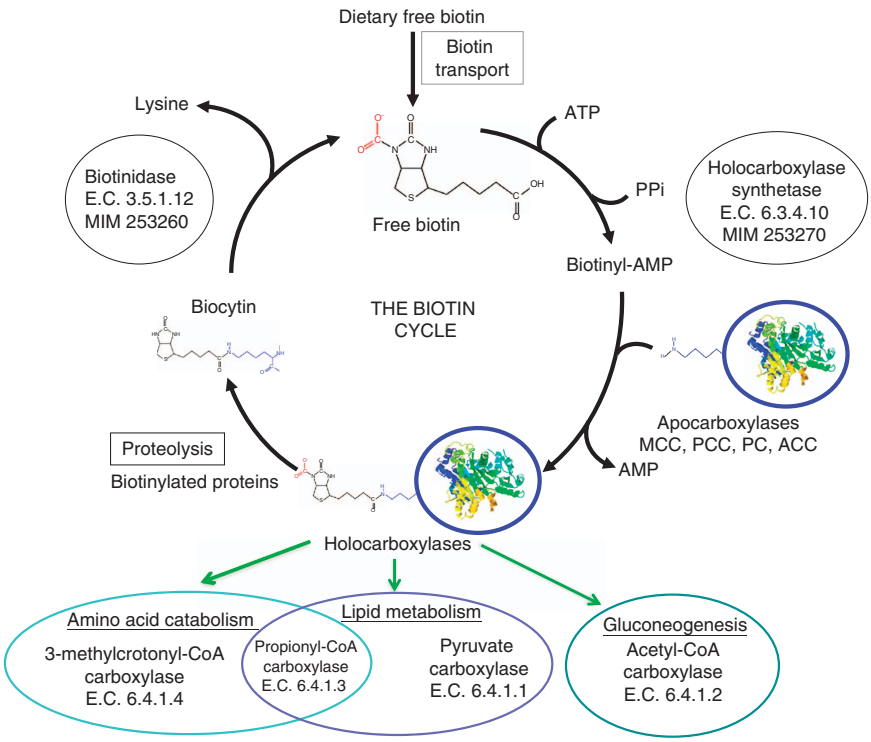

Figure 1 The biotin cycle. Free biotin, whose primary source is the diet, is actively transported across the intestinal membrane and blood–brain barrier. This free biotin pool provides biotin to the various apocarboxylases (pyruvate carboxylase, acetyl-CoA carboxylase, propionyl-CoA carboxylase, and beta-methylcrotonyl-CoA carboxylase). Holocarboxylase synthetase assembles active holocarboxylases by covalently binding biotin to their active sites. The carboxylases are important for gluconeogenesis, fatty acid synthesis, and the catabolism of several branch-chain amino acids. Eventually, the holocarboxylases are degraded proteolytically to biocytin or small biotinyl-peptides. These compounds are further cleaved by biotinidase to produce lysine and free biotin, which can enter the free biotin pool, thereby recycling the vitamin. ACC, acetyl-CoA carboxylase; AMP, adenosine monophosphate; ATP, adenosine triphosphate; MCC, 3-methylcrotonyl carboxylase; PC, pyruvate carboxylase; PCC, propionyl-CoA carboxylase; PPi, inorganic pyrophosphate.

and "Biotinidase Deficiency and *BTD*," hosted by ARUP Laboratories (http://www.arup.utah.edu/database/BTD/BTD_welcome.php). Over 200 biotinidase variants are consolidated in the ARUP database, with more than 150 categorized as pathogenic; missense changes comprise 145 of the 204 variants listed.[13–15] Four common pathogenic variants cause profound biotinidase deficiency.[16] Among children ascertained because of clinical symptoms, the two most commonly reported variants are a seven-base deletion/three-base insertion (c.98_104delinsTCC) in exon 2, occurring in at least one allele in approximately 50% of individuals, and p.Arg538Cys in exon 4, occurring at least once in 30% of children.[17,18] Each of these variants result in complete absence of biotinidase protein. Other relatively common variants discovered by newborn screening are p.Gln456His, associated with profound deficiency, and p.Asp444His, a substitution that reduces enzymatic activity by about 50%.[19,20] The p.Asp444His variant in *trans* with a severe *BTD* pathogenic variant is associated with partial biotinidase deficiency, while in *cis* with p.Ala171Thr (i.e., as the double mutant p.[(Ala171Thr);(Asp444His)]), results in a profound biotinidase deficiency allele.[21] Sequencing of *BTD* can be targeted to the most common disease-causing variants or comprehensively analyzed by forward and reverse sequencing of the exons and flanking intronic regions. Full-gene sequencing is more common to confirm status and will likely be used more as the cost of testing decreases. A gene card published in the *European Journal of Human Genetics* offers considerations for molecular testing.[22,23]

Initially, most symptomatic children with biotinidase deficiency were found to have 3% of mean serum biotinidase activity of normal individuals.[24] Three standard deviations above this mean, corresponding to 10% of mean normal activity, was taken as the threshold below which individuals were considered to have profound biotinidase deficiency. After newborn screening for biotinidase deficiency was introduced, babies were identified with about 25% of mean normal activity.[25] Ultimately, it was determined that essentially all of these children had the p.Asp444His variant as one of their alleles, with the resultant aberrant enzyme contributing about 50% of normal activity.[20] This variant, together with a variant for profound deficiency on the other allele, results in 10–30% of mean normal biotinidase activity.[26] These children are considered to have partial biotinidase deficiency. Because biotinidase deficiency is not due to a continuous spectrum of residual activity but rather to a biphasic distribution of activity, the terms "profound biotinidase deficiency" and "partial biotinidase" are useful functional descriptions.

Clinical description of disease

The initial clinical presentation and ultimate expression of profound biotinidase deficiency are quite variable, even within the same family.[6,27] Symptoms in untreated patients usually appear between 2 and 5 months of age, but may not be evident until several years of age.[28] Abnormalities involving the central nervous system are frequently the first features to occur.[29] More than 70% of clinically ascertained children exhibit seizures, hypotonia, skin rash, or alopecia at some time prior to diagnosis and treatment.[30] About half of the children have ataxia, developmental delay, conjunctivitis, and visual problems, including optic atrophy.[31] Over three-quarters of symptomatic children develop hearing loss.[32] The hearing loss, visual abnormalities and degrees of developmental delay seem to be irreversible once they occur, even following initiation of biotin therapy.[33] Affected individuals may have features ranging from multiple mild episodes of seizures and ataxia, to severe metabolic compromise, which can result in coma or death.[27] Some children with profound biotinidase deficiency developed symptoms only later in childhood or during adolescence.[34,35] They exhibited motor limb weakness, spastic paresis, and eye problems, such as loss of visual acuity and scotomata, rather than the more characteristic symptoms observed in younger untreated children with the disorder.

There are several reports of adults with profound biotinidase deficiency who were ascertained because they had children with profound biotinidase deficiency identified by newborn screening. These adults were asymptomatic despite having never been treated with biotin.[36–38] Several of these parents had extensive evaluations, including rigorous dietary histories, but they failed to demonstrate any clinical or biochemical features. None of these adults had null variants, but all had enzymatic activities in the profoundly deficient range with some residual activity. The explanation as to why they have remained asymptomatic is unknown. In addition, a number of adolescents and adults with profound biotinidase deficiency have presented with myelopathy, with or without vision abnormalities.[33,35,39,40]

Biochemically, untreated individuals may exhibit metabolic ketoacidosis, lactic acidosis, and/or hyperammonemia.[24] Other metabolic abnormalities are more variable and may include elevated excretion of 3-hydroxyisovaleric, lactic, and 3-hydroxypropionic acids and 3-methylcrotonylglycine by urine organic acid analysis, as well as mildly elevated 3-hydroxyisovalerylcarnitine (C5-OH) by plasma acylcarnitine analysis.[41] These metabolic abnormalities are variable, and affected children, whether symptomatic or asymptomatic, do not always exhibit ketoacidosis or organic aciduria.[24]

Biotin supplementation appears to prevent the development of symptoms in presymptomatic children with profound biotinidase deficiency.[42,43] Furthermore, all symptomatic children with profound deficiency who have been treated with pharmacological doses of biotin (5–20 mg daily) have shown clinical improvement.[27] Seizures and ataxia resolve within hours to days and the cutaneous manifestations usually resolve within weeks. Depending on the severity and frequency of episodes of metabolic and neurological compromise, many children with developmental delay rapidly achieve new milestones or regain those that were lost. Children with biotinidase deficiency, who have failed to comply with their biotin therapy, either unintentionally or deliberately, developed symptoms within several weeks to months. These cases illustrate the importance of early diagnosis and initiation of life-long treatment. A recent study reported 44 older

adolescents and adults, ages 16 to 32 years old, with profound biotinidase deficiency ascertained by newborn screening with excellent outcomes.[40]

Partial biotinidase deficiency, defined as 10–30% of mean normal serum activity, initially was considered a variant without clinical consequences. Later, several reports described children with partial biotinidase deficiency who developed symptoms under stress, such as an infection or starvation.[26,44] The symptoms resolved with biotin therapy. The increased susceptibility to developing symptoms in some individuals with partial biotinidase deficiency is not fully understood. There is no consensus within the field regarding treatment of individuals with partial biotinidase deficiency. Several studies have demonstrated excellent outcomes for treated children with profound or partial biotinidase deficiency.[43,45,46] A large retrospective study reviewing clinical histories of individuals with profound (22) or partial (120) biotinidase deficiency identified by newborn screening supports the long-term benefit of treatment and management of both populations.[47]

Five individuals have been found with elevated K_m values, causing reduced binding affinity for the colorimetric assay substrate, biotinyl-*p*-aminobenzoate.[48,49] Two of these individuals had profound biotinidase deficiency; the other three had activities in the partial biotinidase range. The first case was identified symptomatically, whereas the other four were found as part of newborn screening follow-up. An individual with a K_m variant is potentially more likely to become symptomatic if metabolically stressed.[48] If only individuals with profound biotinidase deficiency are treated, those with partial activity and a K_m variant could be placed at risk for developing symptoms. Conservatively treating all individuals with profound or partial biotinidase deficiency would likely prevent symptoms in all variants.

Newborn screening for biotinidase deficiency

Methods for the semiquantitative determination of biotinidase activity on dried blood spots were developed in 1984, and first used that same year in a pilot newborn screening program in Virginia.[50] Biotinidase deficiency meets many of the criteria for inclusion in state newborn screening programs: there is an inexpensive and reliable screening test, the disorder is associated with high morbidity and mortality if untreated, an easy and effective treatment exists, and the population incidence is comparable to that of other disorders screened.[51] Several studies have shown the cost-effectiveness of screening for both profound and partial biotinidase deficiency.[52–54] Currently, all newborn screening programs in the United States and more than 30 other countries screen for biotinidase deficiency, with multiple recent studies suggesting that additional countries are considering incorporation of biotinidase deficiency into their newborn screening programs.[55–60] Historically, the screening method used was a colorimetric assay of biotinidase activity measured in dried blood spots, and states have individually established their own screening cutoffs and rescreening or follow-up protocols.[50] Commercial kits based on fluorescence are now available, and at this time no data are available regarding the frequency of use of the different assays across the United States. Some states report qualitative screening results as either positive or negative, whereas others report quantitative enzyme activities with a screening cutoff value. The newborn screening tests should not be used to discriminate between profound and partial biotinidase deficiency.

Fluorescence-based biotinidase assays use artificial substrates in these kits: 4-methylumbelliferyl biotin, 6-aminoquinoline, or europium-labeled biotin (per information provided by Perkin-Elmer in the package insert of GSP Neonatal Biotinidase Kit).[61,62] Interfering compounds have been noted, some of which include ampicillin, bilirubin, hemoglobin, and glutathione.[62] A published comparison suggests that the fluorescence method using biotinyl-6-aminoquinoline as the substrate was slightly more specific and sensitive than the colorimetric assay.[63] Further studies are warranted to compare the various methods used for newborn screening.

Another advance in newborn screening methods includes multiplex plate testing that may facilitate expanding the number of enzymes assessed at one time.[61] Szabó and colleagues recently published a liquid chromatography–tandem mass spectrometry based method that involves pretreating filter cards with *N*-biotinyl-*p*-aminobenzate to address delayed sample processing.[64] The expansion of available technologies may generate confusion when comparing enzyme activity reports between laboratories. The variety of methods allows newborn screening programs to choose a test most appropriate for their particular challenges, whether specimen volume, processing time, or cost.

Because biotinidase deficiency screening is performed by direct enzyme assay, results are not influenced by dietary protein intake, as is the case for phenylketonuria or other amino acidopathies. Approximately 50% of false-positive results (i.e., low estimates of enzyme activity) are due to prematurity and most others to mishandling of samples and possibly their exposure to excessive heat and/or humidity.[65,66] The effect of transfusion on biotinidase screening has not been established. Because red blood cells do not express biotinidase activity, it may be helpful to determine whether the patient was transfused with whole blood or packed red blood cells. However, variable, but possibly significant, quantities of plasma/serum are retained in the preparations of packed red blood cells and the "circulating life" of biotinidase in these preparations is unknown. One approach for determining the child's actual enzyme status following transfusion is to measure biotinidase activities in the parents. If both parents are clearly in the normal range, then it is unlikely that the child has profound or partial deficiency. DNA studies in this scenario may resolve the issue. It is also possible to provide the infant with biotin supplementation for several months before repeating the confirmatory testing, since this treatment does not affect performance of the biotinidase assay. In general, the parental enzymatic results are reassuring.

Conditions identified by enzymatic biotinidase testing

The enzymatic determination of biotinidase activity identifies individuals with profound biotinidase deficiency (less than

10% mean normal serum activity) and those with partial biotinidase deficiency (10–30% of mean normal serum activity).[26] Heterozygous individuals may also be identified by this test as having approximately 50% of mean normal activity, although there may be significant overlap among the various groups. At least some of the variability arises from clinical status, assay interferents, and sample-handling artifacts (see below). Before a diagnostic category can definitively be assigned, the laboratory must establish ranges from known affected individuals and obligate heterozygotes. In addition, all preanalytical and analytical variables must be carefully controlled before a measured decrease in activity can be attributed to an inherited defect in biotinidase.

Incidence

Based on newborn screening outcome data from 2006, the incidence of profound biotinidase deficiency in the United States is estimated at 1/80,000 births, and that of partial biotinidase deficiency between 1/31,000 and 1/40,000 (http://genes-r-us.uthscsa.edu/sites/genes-r-us/files/resources/genetics/2006datareport.pdf), although frequencies may vary within and between states by local population. Earlier studies indicated similar findings, with the overall incidence of biotinidase deficiency estimated at 1 in 60,000 newborns worldwide, equally divided between profound and partial biotinidase deficiency.[55] The majority of reported patients who were ascertained clinically have been of European descent.[56] The disease incidence varies between countries, with incidences in Brazil as high as 1 in 9,000 and possibly higher incidences in countries with a high degree of consanguinity, such as Turkey, Saudi Arabia, and the United Arab Emirates.[57,58,67–70]

Mode of inheritance

Biotinidase deficiency is inherited as an autosomal recessive trait.[5]

PREANALYTICAL REQUIREMENTS

Sample types

Biotinidase activity is reliably measured in serum and plasma; activity can less readily be determined in fibroblasts, leukocytes, and other tissue extracts.[71] Biotinidase activity can also be measured in cultured amniocytes, although in practice this is seldom performed because of the treatability of the disorder.[72] Plasma or serum samples may not be suitable for individuals who have undergone transfusion; in such cases, determining the carrier status of the parents may be an alternate strategy. Samples collected postmortem are not suitable for enzyme assay; in such cases, the diagnosis may be established via molecular testing or by enzymatically determining the carrier status of the parents. Molecular studies for *BTD* variants are performed on DNA isolated from leukocytes, fibroblasts, or dried blood spots.

Sample volumes

Specific requirements of sample type, collection volumes, and conditions of sample shipping and handling must be established by the laboratory and made available to referring physicians upon request. As a general guideline, 1–2 ml of serum or plasma is required for biotinidase testing. Approximately 2–3 ml of whole blood should be collected by venipuncture in a red-top or green-top (sodium or lithium heparin) tube, and the cells separated from serum/plasma by centrifugation within one hour of sample collection. Samples should be immediately frozen at –80 °C, and remain frozen at this temperature until the time of testing. When testing is performed following an abnormal newborn screen, blood should also be collected from the parents and sent with the patient's sample to aid in the interpretation of results. Similarly, a sample obtained from an unrelated control is useful for distinguishing true biotinidase deficiency from decreased activity due to sample handling artifacts. DNA testing typically requires 1–2 ml whole blood collected in a lavender-top (EDTA) tube.

Conditions of sample shipping, handling, and storage

Blood spots collected for newborn screening must be dried completely prior to shipping to the laboratory because humidity or wet samples result in significant loss of enzyme activity. Serum or plasma samples for diagnostic assays must be frozen and shipped to the laboratory on dry ice. Storage of samples at –20 °C has been shown to lose enzyme activity over time; long-term storage should be at –80 °C for quality control samples (B.W., unpublished data).[73] As noted above, the use of parental and/or unrelated controls is recommended whenever possible, although this may not be operationally feasible in all settings. Samples for DNA testing should be shipped to the laboratory at ambient temperature as soon as possible, but are stable for several days from the time of collection.

METHOD VALIDATION

Calibration and quantitation

The most widespread method for the diagnosis of biotinidase deficiency is based on enzymatic assay using the artificial substrate of biocytin, biotin-4-amidobenzoic acid.[5,50,74] This substrate and all other reagents used in the assay are commercially available, but must be individually prepared and validated by each laboratory before use. Validation of assay performance should be established by assessing inter- and intraday variability, analytical measurement range, and effects of interference. In particular, sulfa drugs are known to interfere and can lead to falsely elevated estimates of activity if not properly controlled (see below). Performance characteristics should be verified on a regular basis as specified by the Clinical Laboratory Improvement Amendments (CLIA).

Reference ranges

Reference ranges for biotinidase activity from individuals with normal activity, intermediate activity (heterozygous for

Table 1 Mean Biotinidase activity for Various Categories of Individuals

Category	Mean Biotinidase activity ± SD (nmol/min/ml serum) (*n*)
Normal individuals	7.57 ± 1.41 (100)
Obligate heterozygotes	3.49 ± 0.72 (21)
Affected individuals (symptomatic)	0.12 ± 0.18 (23)
Affected individuals (newborn screening)	0.19 ± 0.16 (41)
Individuals with partial biotinidase deficiency	1.47 ± 0.41 (23)

Adapted from ref. 2.

profound deficiency), partial deficiency, or profound deficiency are summarized in **Table 1**. Because there may be variability between laboratories, reference ranges must be established by each laboratory, ideally using as many patients as possible in each of the enzymatic categories. Because full-term newborns have 50–70% of mean normal adult biotinidase activity, ideally a separate reference range should be established for this age group.[75] Alternatively, laboratories may elect to establish a broader reference range based on a single large cohort (i.e., 20–50) of previously untested, presumed normal individuals. This broad range can evaluate whether a patient's activity is low but does not distinguish between genotypic subgroups. In most cases, particularly for testing initiated because of newborn screening, it is advisable to confirm abnormal enzyme results with genetic analysis to clarify the biotinidase genotype. Laboratories should periodically verify and update their reference ranges as additional newborns' samples are tested and children with the deficiency are identified.

Testing personnel

Appropriate training and ongoing competency requirements for laboratory personnel performing biotinidase deficiency testing must be established and documented. Testing personnel must satisfy CLIA requirements for high-complexity testing and, at a minimum, have an associate degree in a laboratory science or medical laboratory technology from an accredited institution. Stricter requirements apply in some states.

TESTING FOR BIOTINIDASE DEFICIENCY

Sample preparation

For enzymatic testing, serum or plasma samples should be thawed immediately prior to analysis with no other sample preparation steps required. Information pertaining to the collection, analysis, interpretation, and quality control of molecular studies is given by the American College of Medical Genetics and Genomics Standards and Guidelines for Clinical Genetics Laboratories, Part G (Molecular Genetics) at: http://www.acmg.net/ACMG/Publications/Laboratory_Standards___Guidelines/ACMG/Publications/Laboratory_Standards___Guidelines.aspx?hkey=8d2a38c5-97f9-4c3e-9f41-38ee683bcc84.

Analytical methods

The most widespread method for the diagnosis of biotinidase deficiency is based on an enzymatic assay using the artificial substrate biotin-4-amidobenzoic acid.[5,50,74] In a typical assay, serum is incubated with buffered substrate at 37 °C for 30 minutes and the reaction stopped with trichloroacetic acid. During the reaction, biotinidase in patient serum acts to liberate free *p*-aminobenzoic acid from the artificial substrate. The released *p*-aminobenzoic acid is diazotized with sodium nitrite, and the excess nitrite removed by the addition of ammonium sulfamate. Finally, the diazotized *p*-aminobenzoic acid reacts with *N*-1-naphthyl-ethylene-diamine dihydrochloride to yield a mauve-colored product, which is measured spectrophotometrically at 546 nm. The net absorbance is directly proportional to the amount of *p*-aminobenzoic acid released, which in turn, is directly proportional to biotinidase activity in the sample. This method also forms the basis for newborn screening for biotinidase deficiency, and has been adapted for use with microtiter plates.[50,76]

Biotinidase activity can also be measured using a fluorescent technique with biotinyl-6-aminoquinoline as an artificial substrate.[77] This substrate is more expensive than that for the colorimetric method, but has also been adapted to newborn screening and the testing of dried blood spots.[78] There are other methods for determining biotinidase activity, such as measuring the release of biotin from biocytin, using other fluorescent biotinylated derivatives and radioisotopic biotinylated analogs, but they are more expensive, require more time, are difficult to perform, and often are not readily adaptable to enzymatic determinations using dried blood spots.[71,78–83]

Analyses of urinary organic acids by gas chromatography/mass spectrometry or plasma acylcarnitines by liquid chromatography–tandem mass spectrometry sometimes reveal characteristic abnormalities in patients with biotinidase deficiency. However, this approach is never appropriate as the sole testing modality in individuals suspected of having the disorder because many biotinidase-deficient patients will have normal results by analyte testing and, therefore, be missed. Conversely, metabolic abnormalities that may be compatible with biotinidase deficiency, including elevations of 3-hydroxyisovalerate, 3-methylcrotonylglycine, and 3-hydroxyisovalerylcarnitine, can also be characteristic of other conditions and disease states and, if not confirmed by enzyme assay, can lead to an incorrect diagnosis of biotinidase deficiency. If biotinidase deficiency is excluded by enzyme testing, other disorders in the differential diagnosis must be considered.

Because of the simple organization of the *BTD* gene, follow-up molecular testing by either targeted variant analysis or complete sequencing is possible.[84,85] Molecular testing is particularly useful for differentiating individuals with partial deficiency from those who are heterozygous for pathogenic variants, because almost all children with partial deficiency have the p.Asp444His variant.[20] Common variants, including p.Asp444His, can be readily determined by targeted analysis using DNA extracted from a dried blood spot. If results are

not conclusive after performing targeted variant testing, complete gene sequencing and, when necessary, deletion/duplication analysis are also clinically available. With the establishment of molecular variant databases, variant analysis can often unambiguously determine an individual's enzyme-deficient status. For the reasons stated above and because there are variations in activities and reference ranges among laboratories and occasionally patient activity fails to correlate with parental activities, some clinicians and laboratories suggest that all affected individuals have DNA sequencing. Some states and countries routinely perform variant analysis for all newly diagnosed infants as part of newborn screening confirmatory testing.

Quality control

A positive (abnormal) and normal control should be included with each batch of patient samples. The positive control, representing decreased biotinidase activity, may be prepared by heat-inactivating a large pool of normal serum at 60 °C for one hour. Both sets of controls should be stored as aliquots at –80 °C and thoroughly evaluated prior to use in clinical testing. Tolerance limits for acceptance or rejection of quality control performance should be established and procedures developed for instances where quality control criteria are not met. A quality control designee should perform an ongoing (at least monthly) compilation, review, and sign-off of control values.

For diagnostic evaluations, all samples should be tested for the presence of interfering substances, such as sulfa drugs, which may cause false-negative results.[86] This is readily performed by running a substrate-free blank for each patient sample (i.e., by testing the serum/plasma for positive color development in the absence of substrate). This control is typically not performed for newborn screening assays and, fortunately, sulfa drugs are contraindicated during pregnancy and in neonates.

Proficiency testing

An ongoing proficiency testing program for biotinidase testing must be implemented and documented. An external proficiency testing program for biotinidase deficiency newborn screening is offered through the Newborn Screening Quality Assurance Program of the Centers for Disease Control and Prevention.[87] No external proficiency testing programs for diagnostic testing are currently available. A number of approaches given by the Clinical and Laboratory Standards Institute may be taken, including split sample analysis with a reference or other laboratory and storing aliquots of samples from affected individuals (at –80 °C) and rerunning these samples periodically.[88]

TEST INTERPRETATION AND REPORTING

Interpretation

For tests initiated because of abnormal newborn screening, biotinidase activity $<10\%$ of mean normal activity is indicative of profound deficiency and activity between 10% and 30% of mean normal activity is indicative of partial deficiency. Laboratories should apply these guidelines by comparing a child's activity to the mean normal biotinidase activity established in their laboratory. Decreased enzymatic activity can also be due to prematurity or liver dysfunction, although, if all preanalytical variables are properly controlled, this typically does not lead to misdiagnosis of biotinidase deficiency.[89,90] Biotinidase activity in newborns usually increases over the first days to weeks of life, and those with activities 50–70% of mean normal activity do not usually require retesting at a later date. The most common cause of decreased enzymatic activity is sample mishandling. Therefore, a definitive diagnosis of biotinidase deficiency based on low enzyme activity cannot be made unless these variables are properly controlled. The optimal approach is to determine enzyme activities on samples from an unrelated individual (normal control) and both parents (if available). These samples should be collected simultaneously and sent with the patient's sample for testing. The authors are aware of multiple instances where children were diagnosed with profound biotinidase deficiency by reputable laboratories, but did not have appropriate unrelated or parental control samples for comparison. Several of these children were treated for extended periods with biotin before repeat enzyme testing revealed normal biotinidase activities, initiated as part of a later family study or because clinical symptoms failed to improve. These examples emphasize the importance of obtaining appropriate control samples to avoid misdiagnosis of enzyme deficiency. If there is any question of interpretation, gene sequencing can be used to confirm or exclude the diagnosis (targeted analysis will not identify all the possible variants).

Biotinidase activity in the range of 10–30% of mean normal activity is suggestive of partial biotinidase deficiency. Typically, when the parents are tested, one has about 50% of mean normal activity, indicative of being a carrier for a profound deficiency allele, and the other parent has activity that is about 75% of mean normal activity, indicative of being a carrier for the p.Asp444His variant. It is not uncommon that one or both parents have activities that are not clearly interpretable, such as clearly in the normal range. These cases can be resolved by variant analysis. Occasionally, a parent is found to have partial biotinidase deficiency or who has enzymatic activity in the heterozygous range and is homozygous for the p.Asp444His variant.

Intermediate biotinidase activity (i.e., 50% of normal) could be compatible with either heterozygosity for profound biotinidase deficiency or homozygosity for a variant allele, most often p.Asp444His. Again, evaluation of biotinidase activity in the parents is useful in distinguishing between these possibilities, but still may require variant analysis, possibly targeted analysis for the p.Asp444His variant, to determine definitively their status. When interpreting results for possible carrier or variant status, the laboratories must have sufficient experience with the enzymatic ranges of these genotypic groups and must have proper controls in place.

For patients with clinical symptoms suggestive of biotinidase deficiency, the disorder is excluded by enzyme activity > 10% of normal. Enzyme activity < 10% of normal is consistent with profound deficiency, providing that the proper controls were in place to exclude sample mishandling as the source of decreased activity.[84,91] Confirmation by molecular testing is often useful, but may not be necessary in all cases.

Variant analysis for biotinidase deficiency is readily available and can be useful for resolving cases with inconclusive enzymatic activities. The *BTD* gene is composed of four exons, permitting rapid sequencing of the entire complementary DNA and intron–exon junctions.[10] DNA sequencing is not suitable for newborn screening, but is useful for confirmatory testing to help differentiate between individuals with profound and partial biotinidase deficiency, as well as individuals who are carriers for profound deficiency and those homozygous for a partial deficiency allele such as p.Asp444His. Other cases that can benefit from DNA analysis include variability in enzyme activities reported between different specimens or laboratories, or discrepancies between a patient and parental controls. If variant analysis fails to identify a variant(s) consistent with low enzymatic results, it is possible that the individual has a deletion of the *BTD* gene on one or both alleles.[92,93] Targeted variant testing is less commonly used given the ease of full-gene sequencing and may be most appropriate for family studies or prenatal diagnosis following sequencing of a proband. Optimal sample preparation, handling, and shipping with the inclusion of appropriate control samples can make molecular analysis unnecessary and eliminate the expense of repeated confirmatory testing.[67,88]

There is still little known about phenotype/genotype correlations. However, it is important that all children with profound biotinidase deficiency be treated with adequate biotin therapy.[94] There is one report of symptomatic individuals with null variants causing profound biotinidase deficiency who appear more likely to have hearing loss than symptomatic individuals with other profound deficiency variants.[95] In addition, it has been proposed that children with some residual enzyme activity may have a less severe clinical course than those with no measurable biotinidase activity and a lower requirement for biotin supplementation for treatment.[43] K_m variants have been identified by enzyme studies; however, kinetic studies are not routinely performed and information is lacking about the correlation of these variants with their respective phenotypes. Future studies to determine which DNA changes result in K_m variants will aid in the development of management guidelines for these cases. Our knowledge about phenotype/genotype correlations for biotinidase deficiency may improve as databases grow, combining enzyme activity, clinical descriptions, and DNA sequences.

Reporting

Patient reports must contain appropriate patient and specimen information as contained in Sections C 2.4, 2.4.1, and 2.4.2 of the ACMG Standards and Guidelines for Clinical Genetics Laboratories (http://www.acmg.net/acmg/Publications/Standards___Guidelines/General_Policies.aspx) and as specified by CLIA. Written reports should contain biotinidase activity, units of measure, an appropriate reference range, and an interpretation. Recommendations for follow-up, including referral to a metabolic specialist, should also be included when appropriate.

ACKNOWLEDGMENTS

This work is supported in part by the Safra Research Fund (B.W.). The original document was written by Tina M. Cowan, Miriam G. Blitzer, and Barry Wolf and published in 2010. The revised document was reviewed and endorsed by the Biochemical Genetics Working Group of the ACMG Quality Assurance Committee.

DISCLOSURE

E.T.S. and T.M.C. direct clinical biochemical genetics laboratories that run the tests discussed in the current standards and guidelines on a fee-for-service basis. The other authors declare no conflict of interest.

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ERRATUM | Genetics inMedicine

ERRATUM: Laboratory diagnosis of biotinidase deficiency, 2017 update: a technical standard and guideline of the American College of Medical Genetics and Genomics

Erin T. Strovel, PhD, Tina M. Cowan, PhD, Anna I. Scott, PhD, Barry Wolf, MD, PhD; on behalf of the ACMG Biochemical Genetics Subcommittee of the Laboratory Quality Assurance Committee

Genet Med online publication, 14 December 2017; doi:10.1038/gim.2017.201

Correction to: *Genet Med* online publication 6 July 2017; doi:10.1038/gim.2017.84

In the originally published version of this article, sentences in the Acknowledgments section were transposed. The correct text appears below.

ACKNOWLEDGMENTS

The original document was written by Tina M. Cowan, Miriam G. Blitzer, and Barry Wolf and published in 2010. This work is supported in part by the Safra Research Fund (B.W.). The revised document was reviewed and endorsed by the Biochemical Genetics Subcommittee of the ACMG Laboratory Quality Assurance Committee.

The publisher regrets the error.

Genetics in Medicine (2023) 25, 164-165

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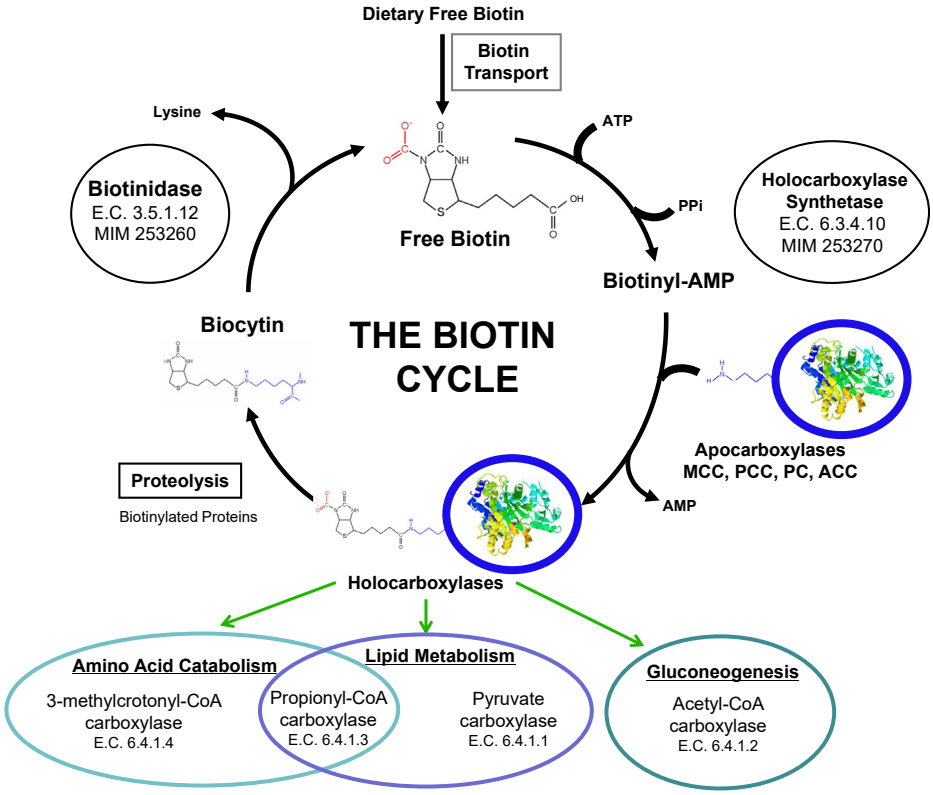
CORRECTION

Laboratory diagnosis of biotinidase deficiency, 2017 update: a technical standard and guideline of the American College of Medical Genetics and Genomics

Correction to: *Genetics in Medicine* 2022; https://doi.org/10.1038/gim.2017.84

In the article “Laboratory diagnosis of biotinidase deficiency, 2017 update: a technical standard and guideline of the American College of Medical Genetics and Genomics” by Strovel ET et al (*Genet Med* 2017;19:P1-10), there was an error in Figure 1. The labels "Acetyl-CoA carboxylase E.C. 6.4.1.2” and “Pyruvate carboxylase E.C. 6.4.1.1” were placed incorrectly at the bottom of the figure artwork. See revised Figure 1 shown on next page.

doi: https://doi.org/10.1016/j.gim.2022.11.014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E.C. 6.3.4.10
MIM 253270
Biotinyl-AMP
Biocytin
THE BIOTIN CYCLE
Apocarboxylases
MCC, PCC, PC, ACC
AMP
Proteolysis
Biotinylated Proteins
Holocarboxylases
Amino Acid Catabolism
3-methylcrotonyl-CoA carboxylase
E.C. 6.4.1.4
Lipid Metabolism
Propionyl-CoA carboxylase
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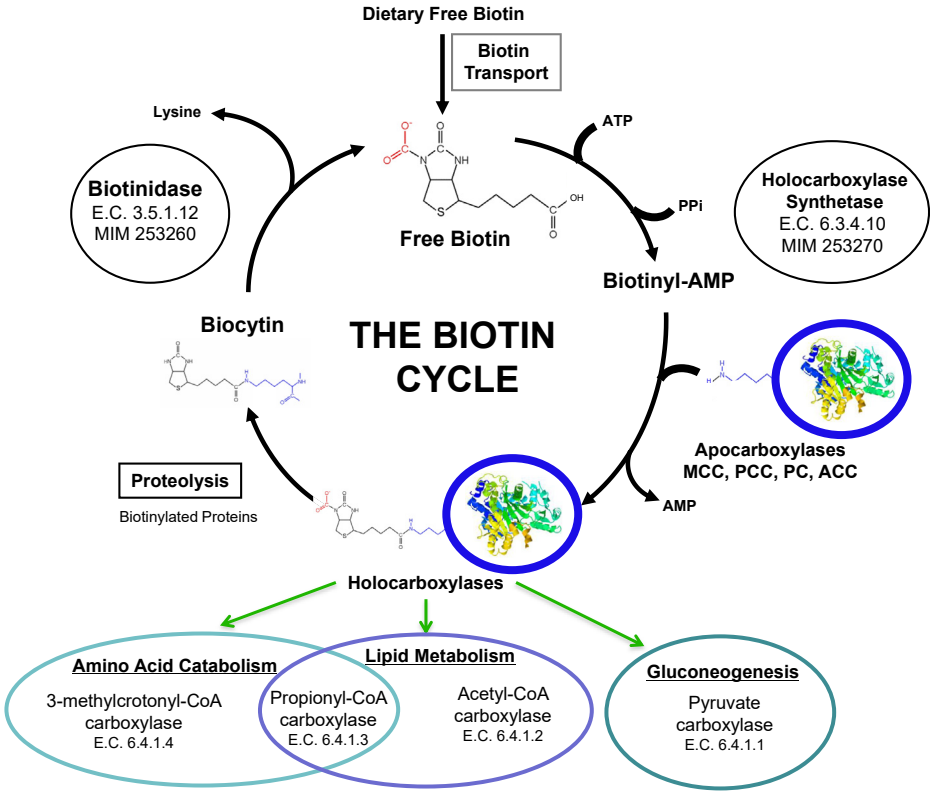
Gluconeogenesis
Acetyl-CoA carboxylase
E.C. 6.4.1.2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Apocarboxylases
MCC, PCC, PC, ACC
AMP
Proteolysis
Biotinylated Proteins
Holocarboxylases
Amino Acid Catabolism
3-methylcrotonyl-CoA carboxylase
E.C. 6.4.1.4
Lipid Metabolism
Propionyl-CoA carboxylase
E.C. 6.4.1.3
Acetyl-CoA carboxylase
E.C. 6.4.1.2
Gluconeogenesis
Pyruvate carboxylase
E.C. 6.4.1.1