Original Article
DOI: https://doi.org/10.70749/ijbr.v3i10.2372

INDUS JOURNAL OF BIOSCIENCE RESEARCH
https://ijbr.com.pk
ISSN: 2960-2793/ 2960-2807

A Comprehensive Study of *BTD*: Total Reported Variants, *In-silico* Analyses and Overview of Functional Studies

Qari Muhammad Iqbal[1], Zeenia Iqbal[2], Samreen Iqbal[3], Muhammad Talha Aftab[4], Areesha Rashid[5]

[1]Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Punjab, Pakistan.
[2]Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, Punjab, Pakistan.
[3]National Centre for Bioinformatics, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan.
[4]Department of Food, Environmental and Nutritional Sciences, University of Milan, via Celoria 2, Milan, Italy,
[5]Centre of Excellence in Molecular Biology (CEMB), Quaid-i-Azam Campus, University of the Punjab, Lahore, Punjab, Pakistan.

ARTICLE INFO

Keywords: Biotin Metabolism, Biotinidase Deficiency, *BTD* Variants, Functional Study of *BTD*, *In-silico analysis*, Pathogenicity.

Correspondence to: Areesha Rashid, Centre of Excellence in Molecular Biology (CEMB), Quaid-i-Azam Campus, University of the Punjab, Lahore, Punjab, Pakistan.
Email: areesha.rashid@bs.qau.edu.pk
ORCID: https://orcid.org/0009-0006-0401-2174

Declaration

Authors' Contribution

QMI: Study design, collected and interpreted the data and performed *in-silico* analyses. **ZI:** Assisted in data interpretation. **SI:** Edited the manuscript and improved readability. **MTA:** Reviewed the accuracy of references and technical details. **AR:** Finalized and approved the manuscript.

Conflict of Interest: No conflict of interest.

Funding: No funding received by the authors.

Received: 03-08-2025 Revised: 28-09-2025
Accepted: 02-10-2025 Published: 10-10-2025

ABSTRACT

BTD encodes the biotinidase enzyme, responsible for recycling and maintaining biotin homeostasis in the human body. Biotin is a water-soluble micronutrient essential for various metabolic processes, with most being recycled by the biotinidase enzyme under normal physiological conditions. The process involves Holocarboxylase synthetase covalently attaching free biotin to Apocarboxylases, such as pyruvate carboxylase, 3-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase, and acetyl-CoA carboxylase, forming active Holocarboxylases. These active forms are then proteolyzed into biocytin and/or biotin peptides, which are subsequently cleaved by biotinidase enzyme, thus completing the biotin recycling loop. Variants within *BTD* disrupt the catalytic activity of biotinidase, leading to an inability to recycle biotin. Biotinidase deficiency, an autosomal recessive inherited metabolic disorder, can result from this disruption, causing the accumulation of biotin metabolites and subsequent damage to the peripheral and central nervous systems. The objective of this study was to analyze *BTD* variants and assess their structural, functional, and clinical significance in biotinidase deficiency. This study presents a comprehensive analysis of *BTD* variants, identifying a total of 740 reported variants, with exon 4 being a significant hotspot with 452 variants, indicating its potential importance for future genetic screening and diagnostic strategies. The research further provides an in-silico analysis of the BTD proteins, detailing their pathogenicity, domain structure, conserved regions, and key amino acids involved in interaction and structural integrity. Functional studies utilizing animal models demonstrate that *BTD* knockout adversely affects physiological features and metabolic pathways, with these effects being reversible upon biotin supplementation.

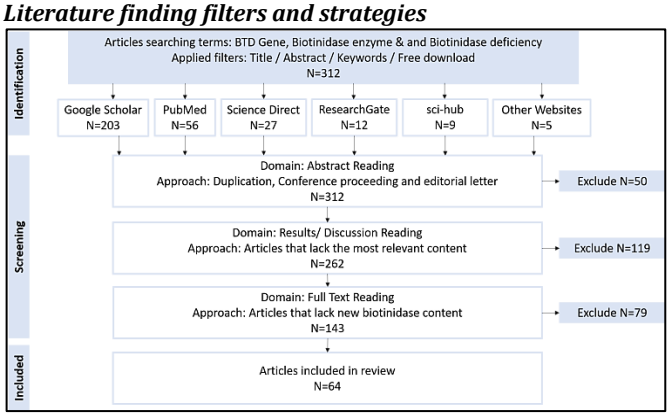
INTRODUCTION

Genetic disorders impose a considerable health burden in low- and middle-income countries [1]. These result from variations in genes, DNA, or chromosomal material, leading to a range of symptoms associated with specific diseases [2]. *BTD* plays a crucial role in human metabolism by encoding the enzyme biotinidase, which is responsible for recycling biotin, a water-soluble B vitamin essential for activating biotin-dependent carboxylases involved in key metabolic pathways such as gluconeogenesis, fatty acid synthesis, and amino acid catabolism [3]. *BTD* variants include numerous mutations over 165 reported, which comprise missense, nonsense, splice-site, and frame shifting indels distributed across the gene without a mutation hotspot. These mutations alter enzyme activity, leading to either profound or partial biotinidase deficiency, with clinical phenotypes ranging from severe neurological impairment to milder metabolic disturbances [4]. Research highlights the use of in silico tools and functional assays to predict the impact of novel variants on enzyme function, facilitating diagnosis, genotype-phenotype correlations, and personalized medicine approaches [5].

Literature Finding Strategies

Following research database websites, filters and strategies were used to find the literature review related to *BTD* in this review article, as shown in figure 1.

Figure 1:
Literature finding filters and strategies



BTD

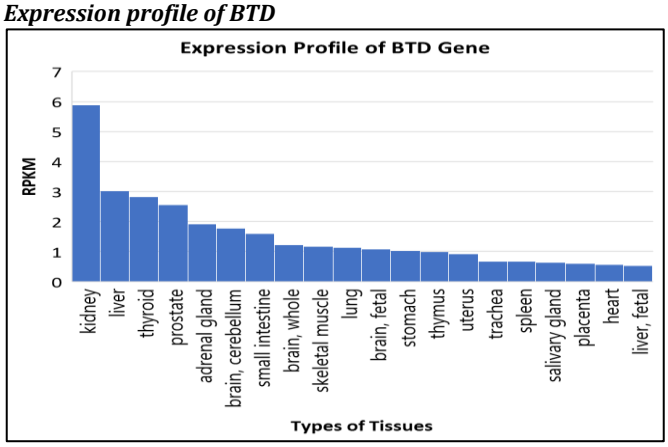
BTD is located on chromosome 3p25 and contains four exons and three introns. The sizes of the exons 1, 2, 3, and 4 are 79 bp, 265 bp, 150 bp, and 1502 bp, respectively, while introns 1, 2 and 3 are >12.5 kb, 6.2 kb, and 0.7 kb. The cDNA of *BTD* encodes a polypeptide of 543 amino acids, with a molecular weight of 61,133 Da [6, 7]. There are two potential AUG start codons in *BTD*, both of which are in the same reading frame. They encode two putative signal peptides, each consisting of 21 and 41 amino acids, respectively. The first potential translational codon is located in exon 1, and the second is located in exon 2. The three initiator (INR) sequences and six consensus methylation sites are considered necessary for the transcription initiation of TATA-less promoters of *BTD* [6].

BTD encodes an enzyme called biotinidase, a glycoprotein or monomeric enzyme. Biotinidase is a key enzyme involved in cleaving the biotin vitamin from dietary protein-bound sources and biocytin and releasing free biotin through a cascade of reactions, which is called biotin turnover [8]. Without this enzymatic action, biotin is lost through feces and urine without being absorbed by the body [9]. First, in 1984, Heard et al. described a method to measure biotinidase activity using a dried blood spot (DBS) card by colourimetric assessment. Biotinyl-p-aminobenzoate is used as a substrate in an enzymatic reaction for a colourimetric assay [10, 11]. Furthermore, commercial ELISA kits, fluorometric assays, radioimmunoassay, digital microfluidic enzyme assays, and molecular genetic analyses are also available on the market for screening BTD deficiency in newborns [12, 13].

Expression Profile of *BTD*

BTD is expressed in multiple tissues; its mRNA is detected via Northern blot analysis in various tissues, including serum, placenta, brain, heart, liver, lung, kidney, skeletal muscle, leukocytes, fibroblasts, and pancreas [6, 9, 14, 15]. *BTD* is also present in several parts of the brain, including the lower auditory brainstem nuclei, the red nucleus, and cerebellar Purkinje cells. *BTD* expression is relatively lower during the early developmental stages as compared to later ages [6]. According to the NCBI database (https://www.ncbi.nlm.nih.gov/gene/686), *BTD* exhibits a distinct expression profile, as illustrated in figure 2.

Figure 2:
Expression profile of BTD

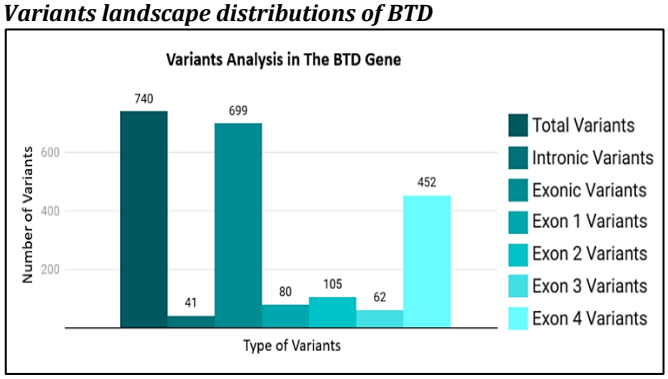


Total Reported Variants in *BTD*

BTD variants have been reported worldwide; however, Turkey is one of the countries where the mutation rate is particularly high [16]. The most common variant reported globally in *BTD* is p.D444H, located in Domain B [6, 17]. Insertion, deletion, and nonsense mutations in biotinidase completely disrupt the enzyme activity; in contrast, the biotinidase enzyme shows some activity in missense mutations [18]. Mutations in the C-terminus of the protein are associated with severe loss of *BTD* activity, while mutations in the carboxy-terminal of the gene cause profound deficiency [6].

To explore the variational landscape of *BTD*, all variants were retrieved from the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/). Applying filtering to the retrieved Excel sheet to categorise the variants, the result shows that the total number of reported variants in *BTD* is 740, as shown in figure 3. These variants were roughly categorised as exonic variants (699) and intronic variants (41). Remarkably, the exonic variants are quite large, which emphasises the pathogenic significance of the coding parts of *BTD*. Further analysis of exonic variants reveals that Exon 4 contains the largest number of variants (452), while Exon 3 has the fewest (62). The variants landscape analysis is helpful in future to set a priority target for diagnosing and screening of biotinidase deficiency, which will save both time and cost.

Figure 3:
Variants landscape distributions of BTD



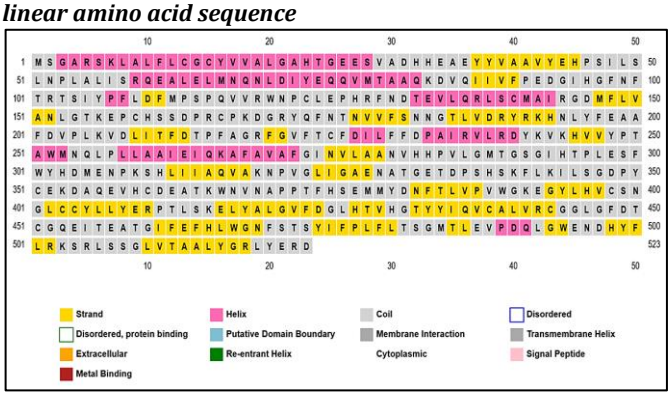
Secondary Structure Analysis of the BTD Protein

The secondary structures of the BTD protein were predicted by the PSIPRED web server

(https://bioinf.cs.ucl.ac.uk/psipred/) as shown in figure 4. According to the PSIPRED prediction, the BTD protein has 18.36% alpha helix, 26% extended strand, and 55.64% coil. These secondary structures play an important role in the overall structure of a protein and its folding [19]. This PSIPRED prediction was subsequently employed to enhance the 3D model of the protein by representing the involvement of each amino acid in protein structure. With the help of this result, any identified variant in *BTD* can be checked to see which structure of the BTD protein will be disrupted by that specific variant.

Figure 4:

The secondary structure prediction of the BTD protein by linear amino acid sequence

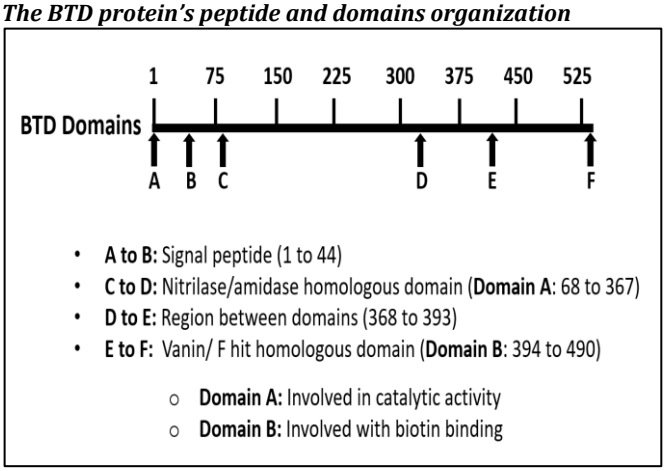


Peptides and Domains Analysis of the BTD Protein

The BTD protein domains and peptides analysis was done by using the UniProt web server (https://www.uniprot.org/uniprotkb/P43251/entry). Corresponding to the signal peptide, the BTD protein has many peptide regions. Which translation initiation site is used depends on these two unique signal peptides. A cellular secretory motif most consistent with the second shorter signal peptide. The BTD protein has two domains, designated as Domain A and Domain B, and a connecting region between them. Domain A (68-367) is the nitrilase/amidase homologous domain involved in the catalytic activity. Any change in this domain would have a potential effect on enzyme activity. Domain B (394-490) is a vanin/ F hit homologous domain involved in biotin binding, as shown in figure 5 [20, 21].

Figure 5:

The BTD protein's peptide and domains organization



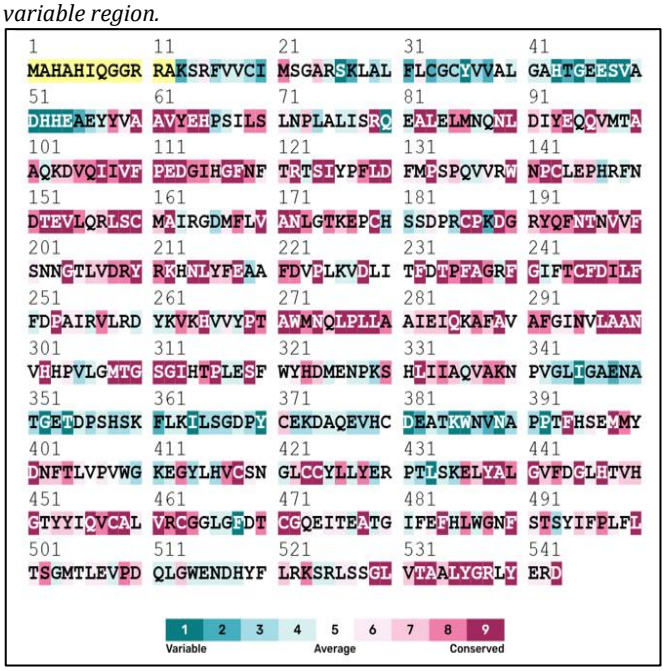
Evolutionary Conservation Prospect of the BTD Protein

When the human BTD protein amino acid sequence was compared with bacterial nitrolases and amidases, it indicated that certain regions in the BTD protein are highly conserved. These conserved regions in the BTD protein comprise the active sites of cysteine in nitrolases and amidases, indicating the site of the BTD involved in the cleavage of the thioester bond between biotin and biocytin [6]. Conservation of the amino acid residues in the BTD protein is measured by the ConSurf web server (https://consurf.tau.ac.il). According to a colour-coding scale, all amino acids are coloured by their degree of conservation, with turquoise indicating variable and violet indicating conserved, as shown in figure 6. The confidence scores are calculated based on probabilistic models. The highly conserved positions suggest that these sites are sensitive and essential for protein structure and functions.

Figure 6:

Illustration of evolutionary conservation of the BTD protein amino acid residues

Red colour on the heatmap represents a highly conserved amino acid. And green colour shows that the amino acids lie within a variable region.

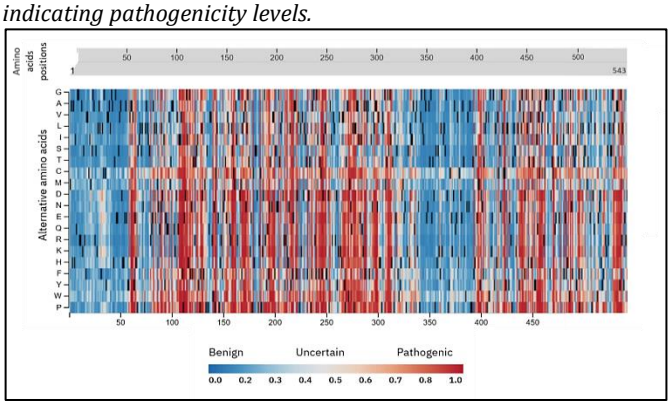


Pathogenicity Analysis of BTD Protein

To assess the possible pathogenic score of alternative amino acids of the BTD protein, the AlphaFold web server (https://alphafold.ebi.ac.uk/entry/) was used. The BTD protein exhibits high pathogenicity, with numerous genetic variants associated with an increased risk of biotinidase deficiency. The structure or function of the BTD protein may be altered due to these variants, leading to dysregulation of *BTD* expression and disruption of normal cellular and metabolic processes. The results show that missense variants, which are highlighted in red on the heatmap, are highly pathogenic, as shown in figure 7. The clinical benefit of this result is that a newly identified variant in *BTD* can be predicted more rapidly

and accurately to see whether that variant is pathogenic or benign.

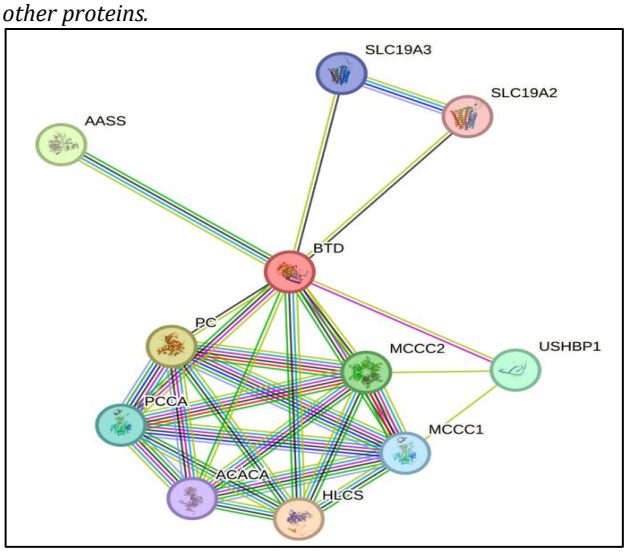
Figure 7:
Pathogenicity analysis of the BTD protein
The y-axis displays a graph of the alternative amino acid makeup of BTD, and the x-axis represents the residue sequence number, indicating pathogenicity levels.



Protein-protein Interaction (PPI)

Protein-protein interaction (PPI) was conducted with the help of the STRING web server (https://string-db.org/). The BTD protein interacts with several biotin-requiring enzymes, including ACACA, PC, MCCC1, PCCA, PCCB, and MCCC2, as shown in figure 8. These enzymes are involved in maintaining homeostasis and various metabolic processes, including fatty acid synthesis, gluconeogenesis, and amino acid catabolism. Thus, the BTD, PPI show the biological significance in cellular metabolism.

Figure 8:
The BTD protein interactions
Proteins are shown as nodes, which are of different colors and the lines (edges) that connect them to the BTD protein and among other proteins.



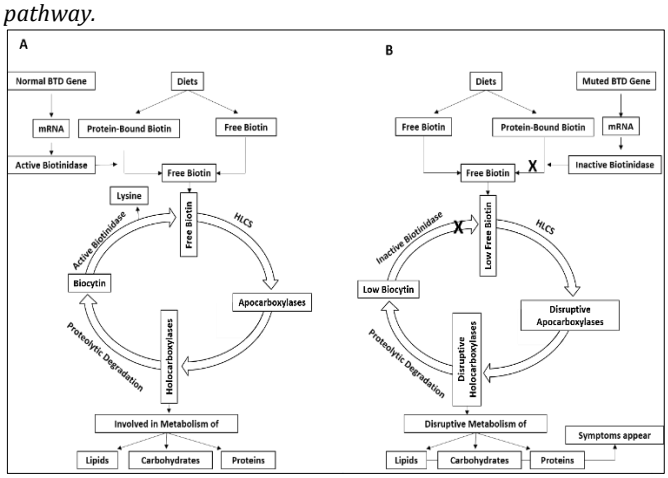
Biotin and Its Sources

Any pathogenic variant in *BTD* can disturb the recycling process of biotin. Biotin is a water-soluble micronutrient also known as Vitamin H, vitamin B7 and coenzyme R [6, 22, 23]. For healthy adults, the recommended daily dietary intake of biotin has ranged from 40 to 60 µg; however, the requirements for biotin during pregnancy may be increased [24, 25]. However, human and other animal cells cannot synthesize biotin. However, yeast, plant, and bacterial cells (Bacillus subtilis, Escherichia coli, Bacillus sphaericus, Kurthia sp., Serratia marcescens, and Pseudomonas mutabilis) can biosynthesise. Biotin is found in all organisms and has been documented in the literature since 1971 [17, 26]. Humans take biotin through an exogenous route [9]. Dietary intake and normal bacterial flora of the large intestine are the primary sources of biotin [15]. Biotin is primarily found in natural foods, including fresh milk, animal liver, fish meat, egg yolk, yeast, mushrooms, soybeans, peanuts, sunflower seeds, broccoli, and sweet potatoes [22, 24, 27].

Cellular, Metabolic and Molecular Roles of Biotin

Under normal physiological conditions, biotin is required in small amounts, as most of it is recycled by biotinidase [28]. Through the catalytic activity of Holocarboxylase synthetase, the free biotin is covalently attached to the Apocarboxylases (pyruvate carboxylase, 3-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase, and acetyl-CoA carboxylase), which use the biotin as a coenzyme, resulting in the formation of active Holocarboxylases. These active Holocarboxylases are subsequently proteolysed into biocytin and/or biotin peptides, which are further cleaved by biotinidase, thereby recycling the biotin, as shown in figure 9 [27].

Figure 9:
BTD pathway
Panel A represents the normal pathway of BTD, which allows effective biotin recycling to activate the carboxylases. Panel B demonstrates the mutant pathway of BTD. The mutant BTD is unable to recycle the biotin; as a result, the activity of carboxylase enzymes is disrupted, which is the main cause of abnormal metabolism and brain calcification. This diagram was adopted from [22] and modified for improved understanding of BTD pathway.



Biotin is primarily distributed among the cytosol fractions and mitochondria, while a small amount is found in the microsomal fractions and in the nuclei (histones) [24, 25]. Biotin plays an important role in the production of proinflammatory cytokines, B and T cell immunity, intracellular signal transduction, cell proliferation and physiology, DNA repair and stabilization, the expression of over 2000 genes, protein modifications, dopamine and ATP production, homeostasis, and prevention of oxidative stress [24, 29-31]. Biotin acts as a coenzyme for carboxylases (methyl crotonyl-CoA carboxylase, pyruvate carboxylase, acetyl-

CoA carboxylase and propionyl-CoA carboxylase) in the mitochondria involved in gluconeogenesis, protein, carbohydrate and lipid metabolism and catabolism of many branch-chain amino acids [15, 18, 22]. Some comprehensive sources, functions, and applications of biotin are described in Figure 10, is adapted from [32].

Biotinidase Deficiency

When biotinidase does not recycle free biotin due to variants [15], a gradual deficiency of free biotin develops in the body, leading to multiple carboxylase deficiency. This deficiency contributes to the abnormal metabolism of lipids, proteins, and carbohydrates, which are the main causes of biotinidase deficiency [33]. Biotinidase deficiency is a rare autosomal recessive inherited metabolic disorder [34], first described by Wolf in 1983 [10]. Biotinidase deficiency is also known as multiple carboxylase deficiency [33]. The global incidence of BD varies from 1:40,000 to 1:60,000 live births [24, 25]. The identification of specific variants in *BTD* in the Pakistani population can significantly contribute to improved diagnosis and management of biotinidase deficiency disease [33, 35].

Classifications and Manifestations of Biotinidase Deficiency

Biotinidase deficiency is classified into two types based on enzyme activity. In partial biotinidase deficiency, biotinidase activity is between 10.1% and 30%, while in profound biotinidase deficiency, biotinidase activity is ≤10%. However, the normal activity of biotinidase is typically exceeded by 66.6% [12, 36]. Profound biotinidase deficiency is further classified into early-onset and late-onset forms [37, 38]. The symptoms of biotinidase deficiency depend on biotinidase activity and other factors, such as exogenous biotin intake and its requirement in metabolic pathways [18]. If profound biotinidase deficiency is left untreated, it can lead to clinical manifestations such as hypotonia, seizures, vision loss, progressive spastic paraparesis, developmental delay, ataxia, optic atrophy, sensorineural hearing loss, respiratory problems, and recurrent infections (including fungal and viral infections) [39, 40]. It can also lead to coma and death [37, 41]. In the case of profound biotinidase deficiency, most symptoms manifest between 1 week and 10 years of age [6]. Most untreated patients may show ketoacidosis, lactic acidosis, eventually hyperammonemia, and elevation of urinary levels of lactate, pyruvate, 3-hydroxyisovalerate and 3-methylcrotonylglycine [42]. While partial biotinidase deficiency has been associated with white matter lesions in the brain [43]. Biotinidase deficiency affects the following main organs and organ systems: the respiratory system (~18%), the auditory system (~27%), the nervous system (~67%), the eye (~34%), the skin (~54%), and the [24, 25].

Newborn Screening Programs and Treatment of Biotinidase Deficiency

In countries like Pakistan, patients with biotinidase deficiency are diagnosed late due to the absence of newborn screening (NBS) programs. NBS programs in any country enable early disease intervention and improved patient-centred outcomes [33, 44]. NBS programs play an important role in public health initiatives worldwide, serving as an indicator of a nation's healthcare development [45]. Biotinidase deficiency is a treatable disease. Patients' clinical symptoms, such as skin rash, alopecia, ataxia, and seizures, usually respond well to the initiation of an oral 20 mg biotin dose once daily, particularly when treatment is started in the early stages [36, 46, 47]. However, developmental delay and hearing loss may not be reversible. Lifelong biotin supplementation is a safe and economical drug [6, 48, 49].

Functional Study of *BTD* in Animal Models

When *BTD* is knocked out in different animals, the following changes were observed. However, some changes in *BTD* knockout animals were improved or restored with biotin supplements, shown in the Table 1.

Table 1: *BTD knockout models and improvements*

Feature/Parameter	In *BTD* knockout models	Improvement with Biotin supplements	Ref.
AMP/ATP ratio	Increases	Improved	[50]
AMPK, AKT and ACC1/2 phosphorylation	Increases		
Free fatty acids synthesis	Increases		
Gluconeogenesis	Increases		
Fatty acid oxidation	Increases		
ABR thresholds	Increases	Improved (if early)	[51]
Sensorineural hearing loss	Increases	No improvement	
Central auditory IPLs (V–I, II–I)	Increases	Improved (if early)	
Ventricular size	Increases	No improvement	[52]
Plasma ammonia	Increases	Improved	[53]
3HIVA and 3MCG (Organic acid)	Increases	Improved	
Electrolytes (Na, K, Cl, HCO_3^-)	Increases	Improved	
Immune cell migration	Increases		[54]
Neuroendocrine markers	Increases		
Neuroendocrine activity	Increases	Improved	
Abnormal brain morphology	Increases		
mTOR phosphorylation	Decreases		[50]
Blood glucose	Decreases		
Triglycerides & cholesterol	Decreases		
GK (Glucokinase)	Decreases		
Myelin and axonal structure	Decreases	Restored	
FAS (Fatty Acid Synthase)	Decreases		
Oligodendrocyte myelination	Decreases	Partially (early)	[51]
Melanocyte-dependent fur colour	Decreases		
Biotin-dependent enzyme levels	Decreases	Restored	
Motor & sensory function	Decreases	Improved	[52]
Carboxylase (PC, PCC, etc.)	Decreases	Improved	
Hepatic biotin content	Decreases	Improved	[53]
Bone Volume	Decreases	Likely improved	
Overall growth (Body weight & length)	Decreases	Improved	[55]
T-cell numbers	Decreases	Improved	
Liver and spleen weight	Decreases	Improved	
Thymus weight & cellularity	Decreases	Improved	
GH Axis (Growth Hormone)	Decreases	likely reported	
Embryo viability	Decreases	Not reported	[56]

DISCUSSION

BTD encodes an enzyme which is called biotinidase. Biotinidase is a key enzyme involved in cleaving the biotin from dietary protein-bound sources and biocytin, releasing free biotin through a cascade of reactions [8]. Biotin is a water-soluble micronutrient also known as Vitamin H, vitamin B7 and coenzyme R [6, 22, 23]. When variants occurred in *BTD*, it did not recycle the free biotin, resulting in low free biotin being entered into the cycle and causing biotinidase deficiency. Biotinidase deficiency is a rare autosomal recessive inherited metabolic disorder in which biotin metabolites are accumulated due to *BTD* variants. These metabolites disrupt the peripheral and central nervous systems [34]

A comprehensive analysis of *BTD* variants was done in this study. Results show that a total of 740 variants were reported, comprising 699 exonic and 41 intronic variants. The number of exonic variants is large, indicating that they are the major cause of biotinidase deficiency. In coding regions, exon 4 has the highest variant 452; this finding helps to target exon 4 first for diagnostic and screening of biotinidase deficiency. Exon 3 has the smallest number of variants and is the target for screening biotinidase deficiency. The advantages of the variants landscape analysis are that, in the future, it will help set a priority target for diagnosing and screening biotinidase deficiency, which will save both time and cost [40, 57]. The PSIPRED result shows the involvement of each amino acid in the BTD protein structure. With the help of this result, any identified variant in *BTD* can be checked to see which structure of the BTD protein will be disrupted by that specific variant. Although the individual alternative amino acid effects were described in previous reports, this study presented the impact of total amino acids of the BTD protein [19, 58]. AlphaFold results demonstrate the pathogenicity of all missense variants of *BTD*. The clinical benefits of AlphaFold results are that any newly identified variant in *BTD* can be predicted more rapidly and accurately to see whether that variant is pathogenic or benign, which is valuable in the scientific community [59, 60]. The results of the Consurf web server predict the conserveness of the BTD protein. The presence of variants in the conserved region will be beneficial for functional studies and evolutionary prospects. Any identified variants in *BTD* can be easily checked using the Consurf result to determine whether they are present in conserved regions or not [61, 62]. Biotinidase enzyme has two domains. Domain A, which is involved in catalytic activity and Domain B, which is engaged in biotin binding activity. The domain analysis results are validated by the NCBI conserved domain and UniProt, which provide scientific justification for the functions of the biotinidase domains [63]. This analysis helps predict the pathogenicity of variants and their severity. If the variant's position falls outside these domains, it means these variants are less likely to be pathogenic for disease [21].

To the best of our knowledge, all functional studies in different animal models of *BTD* were included in this study. The functional research highlights *BTD* knockout models to clarify the various phenomena of the disease and the types of physiological defective features restored by biotin supplements. Previous reports included limited observations, whereas this study combines the effects of the knockout *BTD* with the therapeutic response to biotin. These functional studies prove that the genotype-phenotype correlation is not always constant, and the severity of the disease depends not only on genotype but also on timely treatment. These combined findings not only helped to understand the genetic cause of biotinidase deficiency but also aided in diagnostics for the newborn screening program [64]. Biotinidase deficiency still warrants further investigation in humans, both at the basic research and clinical levels [24, 25].

In the future, targeted sequencing panels will be manufactured that include exon 4. This will enable the proper and timely diagnosis of biotinidase deficiency in countries with limited resources. In-silico results can be validated in the wet lab to verify the protein folding, binding affinities, and pathogenicity. Functional studies in animal models have demonstrated that biotin supplements can correct certain defects, resulting in clinically significant improvements, and the MRI returns to normal within 4 weeks [24, 29]. Therefore, biotin can be effectively utilized as a therapeutic agent in the future.

CONCLUSION

BTD variants are presented in this study from an integrated perspective, and it becomes clear that exon 4 exhibits the highest variability, which is especially important in the pathogenesis of biotinidase deficiency disease. In-silico analysis predicted the pathogenic mechanisms of *BTD* variants, while the functional studies provided practical evidence of disease modelling and therapeutic response. This study consolidates existing knowledge and provides a comprehensive and future-oriented view of *BTD*, which has been previously limited by reports that include computational predictions and functional evidence. It is not only beneficial for the scientific community but also helps in the better diagnosis and treatment of biotinidase deficiency disease.

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