Blue Diaper Syndrome and *PCSK1* Mutations

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abstract

Blue diaper syndrome (BDS) (Online Mendelian Inheritance in Man number 211000) is an extremely rare disorder that was first described in 1964. The characteristic finding is a bluish discoloration of urine spots in the diapers of affected infants. Additional clinical features of the first described patients included diarrhea, inadequate weight gain, hypercalcemia, and nephrocalcinosis. An intestinal defect of tryptophan absorption was postulated as the underlying pathology. However, functional evidence for this theory is lacking. No genetic cause has been identified so far. Here, we report on a boy who presented with neonatal-onset diarrhea, metabolic acidosis, transient hepatopathy, recurrent hypoglycemia, and blue-stained urine spots in his diapers. An ultra-performance liquid chromatography–electrospray ionization–tandem mass spectrometry analysis of urine samples at different time points demonstrated the constant presence of indigo derivatives, thereby confirming the diagnosis of BDS. Of note, the visibility of indigo derivatives in the urine was highly dependent on the urine's pH. To identify the underlying genetic cause of the disease, whole-exome sequencing was performed, leading to the identification of a homozygous frameshift mutation in proprotein convertase subtilisin/kexin type 1 (*PCSK1*; NM_000439.4: c.679del, p.[Val227Leufs*12]). *PCSK1* encodes prohormone convertase 1/3, and mutations within this gene have been reported as a rare cause of early-onset malabsorptive diarrhea and multiple endocrine dysfunction. In our report, we suggest that BDS can be caused by *PCSK1* mutations.

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Dr Distelmaier was involved in the acute patient care, conceptualized and designed the study, and drafted the initial manuscript; Dr Herebian conducted the ultra-performance liquid chromatography–electrospray ionization–mass spectrometry/mass spectrometry analysis, participated in the interpretation of data, and reviewed and revised the manuscript; Dr Atasever was involved in the acute patient care, participated in the interpretation of data, and reviewed and revised the manuscript; Drs Beck-Woedl, Strom, and Haack were involved in the genetic analysis, participated in the interpretation of data, and reviewed and revised the manuscript; Dr Mayatepek participated in the interpretation of data and reviewed and revised the manuscript; and all authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Blue diaper syndrome (BDS) is an enigmatic disease entity that was first described in 1964 in 2 siblings (Online Mendelian Inheritance in Man [OMIM] number 211000). BDS is characterized by the urinary excretion of indole derivatives, leading to the formation of blue-colored indigo.[1] A defect of intestinal tryptophan absorption with consecutive bacterial degradation was postulated. So far, no underlying genetic cause has been identified. Of note, secondary cases of BDS have been reported in the context of fecal colonization with *Pseudomonas aeruginosa* or urinary tract infections.[2,3]

Here, we report on a neonate of healthy consanguineous parents from Syria who presented with malabsorptive diarrhea, metabolic acidosis, hepatopathy, and recurrent hypoglycemia. In addition, a bluish discoloration of the diaper was noted at several occasions, leading to the suspicion of BDS.

CLINICAL REPORT

This boy is the fourth child of consanguineous parents from northern Syria. The siblings and the father are healthy. The mother suffered from recurrent episodes with biliary pancreatitis but is otherwise healthy. The body weights of the parents are within the normal range (mother's BMI: 27.5; father's BMI: 26.1), and there was no family history of endocrine, nutritional, or metabolic diseases.

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The child was born after a normal pregnancy at 41 + 1 weeks' gestation via cesarean delivery (weight: 3580 g; length: 50 cm; head circumference: 37.5 cm; Apgar score 7/9/10; umbilical cord pH: 7.27). Postnatal adaptation was adequate. However, during the second day of life, the boy developed hypoglycemia (14 mg/dL), and intravenous glucose infusion was started. The clinical condition stabilized transiently, and infusion therapy was stopped again. However, during the next days, the child developed diarrhea, and metabolic acidosis (base excess −14 mmol/L) became evident. Transaminases were elevated (glutamic oxaloacetic transaminase: 219 U/L [normal: <79]; glutamic pyruvate transaminase 118 U/L [normal: <48]). In addition, a bluish discoloration of urine spots in his diapers (like ink spots) was repeatedly noticed (Fig 1A). Extensive diagnostic workup, including laboratory investigations with regard to infections or metabolic diseases as well as intestinal and liver biopsies, could not clarify the underlying pathology. Tryptophan levels in plasma were within the normal range (41–65 μmol/L). The results of testing of stool samples for *Pseudomonas aeruginosa* were negative several times. There was no evidence of renal dysfunction or nephrocalcinosis. Serum calcium levels were within normal limits. Because of persistent diarrhea, the placement of a central venous catheter and total parenteral nutrition were required. Subsequently, the bluish discoloration of urine spots in his diapers was noticed less frequently. Starting from the third week of life, oral feeding with an extensively hydrolyzed formula was initiated. This was tolerated only transiently. The subsequent clinical course was complicated by recurrent episodes of hypoglycemia and diarrhea during time periods with enteral nutrition.

At the age of 8 months, the child's condition was stable (body weight: 9.160 kg; 54th percentile), but he still required partial parenteral nutrition. Transaminases normalized, and during the following months, there were no signs of liver dysfunction anymore. An investigation of the corticotropic hormone axis of the patient revealed occasionally low circulating cortisol levels, but an adrenocorticotropic hormone test demonstrated a sufficient cortisol response (maximum: 29.2 μg/dL), thereby excluding hypocortisolism. Adrenocorticotropic hormone levels in the blood were normal. Additional endocrine workup revealed massively increased serum proinsulin levels (330 pmol/L; normal: <11), indicating a problem in peptide hormone processing. The levels of mature insulin during episodes of hypoglycemia ranged from 2.4 to 3.0 mU/L (normal: <2), suggesting altered kinetics of insulin metabolism, which were probably related to the elevated proinsulin levels. Glucagon levels were within the normal range. Moreover, hypothyroidism was diagnosed with elevated thyrotropin levels (maximum: 13.2 μU/mL; normal: 1.36–8.76) and low free thyroxine levels (minimum: 8.3 pg/mL; normal: 10.8–20.3). A substitution therapy with levothyroxine (1×37.5 μg per day) was started. In addition, an investigation of parathyroid hormone revealed moderately reduced levels (1.0 pmol/L; normal: 1.6–6.9) with increased serum phosphate (2.22 mmol/L; normal: 1.15–2.15), suggesting hypoparathyroidism.

At the current age of 12 months, the child still receives an extensively hydrolyzed formula, which is now tolerated better, and stool consistency increased. However, he constantly requires partial parenteral nutrition to avoid episodes of hypoglycemia. During the last 3 months, the boy developed obesity (weight: 11.9 kg [98th percentile; 2.00 *z*-score]; BMI: 20.3 [99th percentile; 2.25 *z*-score]). Up to now, neurocognitive development has appeared to be normal.

ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY–ELECTROSPRAY IONIZATION–TANDEM MASS SPECTROMETRY

One hundred microliters of urine sample were diluted with 900 μL of water (pH 9) and analyzed immediately by ultra-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (UPLC-ESI-MS/MS). The system consisted of an Acquity Ultra-Performance Liquid Chromatography–I Class System (Waters, Altrincham, United Kingdom) coupled with a Waters Xevo TQ-S tandem mass spectrometer, which is equipped with an electrospray ionization source operating in the positive ion mode. Quantitative data were conducted in the multiple-reaction monitoring mode. The chromatographic separation was performed on a Waters Ultra-Performance Liquid Chromatography BEH column C18 (length: 100 mm; ID: 2.1 mm; particle size: 1.7 μm) by using acetonitrile and water (including 5 mM of ammonium acetate; 0.05% formic acid) as mobile phases. A gradient elution was performed, and 5 μL of the prepared samples were injected into the system. The run time and flow rate of this analysis were 6 minutes and 0.4 mL per minute, respectively. For quantitative analysis, the following mass transitions were used for indigo: 263>235 (quantifier ion) and 263>77 (qualifier ion). The compound indigo (internal standard) was purchased from Millipore Sigma (formerly Sigma-Aldrich; Munich, Germany). The compound 3-hydroxyindole that was used for the experiments depicted in Fig 1B was purchased from Santa Cruz Biotechnology.

GENETIC ANALYSIS

Coding regions were enriched by using a SureSelect Human All Exon V6 kit (Agilent Technologies, Santa Clara, CA) followed by sequencing as 100-base pair, paired-end runs on an Illumina HiSeq 4000. Reads

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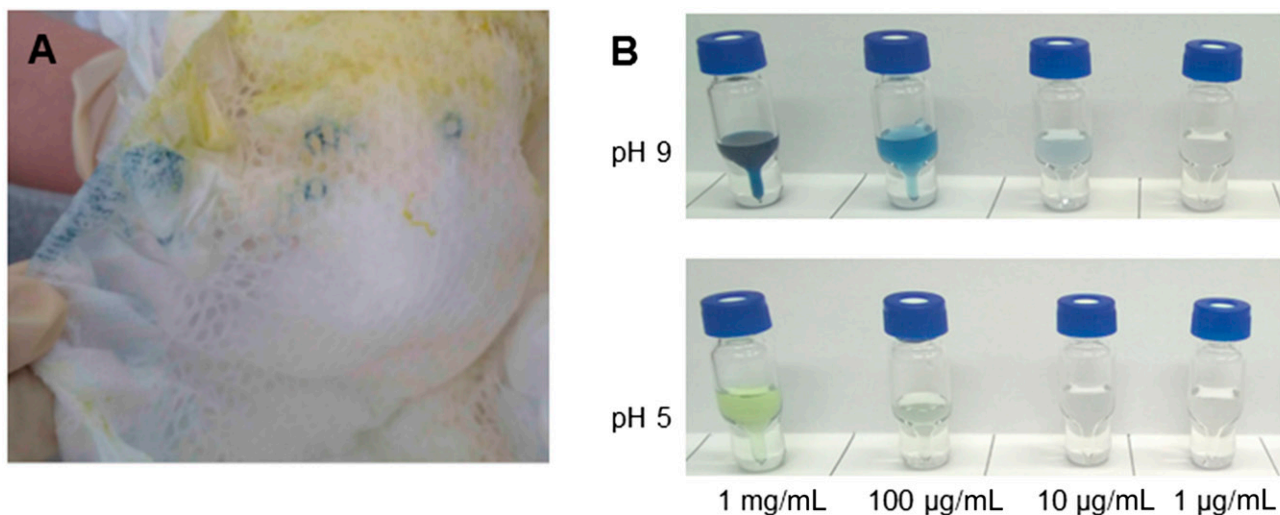

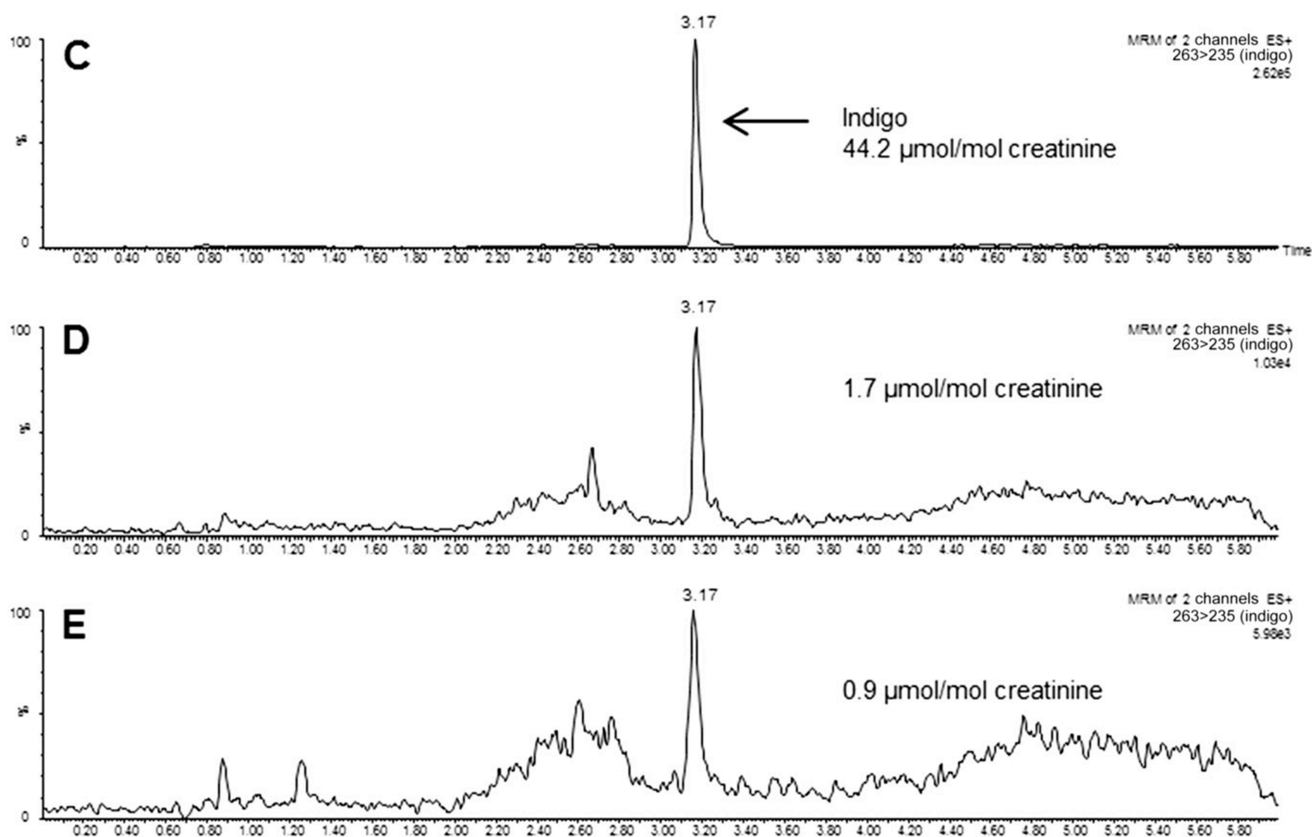

FIGURE 1

A, Bluish discoloration of urine spots in the diaper of the patient. B, Experiment testing the pH dependence of indigo formation. For this purpose, the precursor substance 3-hydroxyindole was used at different concentrations, and pH was adjusted to pH 9 or pH 5, respectively. As depicted, the formation of blue-colored indigo requires an alkaline environment (shown in the upper panel). In an acidic environment, 3-hydroxyindole has a yellowish color that is not visible in urine samples (the lower panel). C–E, UPLC-ESI-MS/MS analysis of the patient's urine samples at different time points. C, Chromatogram demonstrates the presence of indigo in the first week of life. D, Chromatogram demonstrates the presence of indigo at the age of 4 weeks. E, Chromatogram demonstrates the presence of indigo at the age of 10 weeks. For chromatograms depicted in C–E, the concentration values were normalized to creatinine. Of note, the presence of indigo was specific for the patient's urine samples at all time points analyzed and was absent in controls. ES+, electrospray positive mode.

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were aligned to the human reference genome (University of California, Santa Cruz Genome Browser build human genome 19) by using Burrows-Wheeler Aligner version 0.7.5a.[4] Single-nucleotide variants and small insertions and deletions (indels) were detected with SAMtools version 0.1.19.[5] Written informed consent for molecular studies was obtained from the parents. The genetic study was approved by the local ethics committee of the Heinrich-Heine University Hospital (number 4957).

RESULTS AND DISCUSSION

To confirm the diagnosis of BDS, we analyzed urine samples of the child at different time points by using an UPLC-ESI-MS/MS system. Experiments demonstrated the presence of indigo derivatives, with the highest concentrations seen during the first week of life (Fig 1 C–E). Of note, the bluish discoloration of the diapers was variable and not constantly visible. As shown in Fig 1, this might be related to low indigo concentrations, but it also depends on the urine's pH (eg, the formation of indigo from precursor molecules requires an alkaline environment and exposure to oxygen; Fig 1B) as well as the mode of nutrition (eg, during total parenteral nutrition, diarrhea stopped).

To identify the underlying genetic cause of the disease, whole-exome sequencing was performed. We produced 13.9 gigabytes of mappable sequences corresponding to a 155-fold coverage, with >98% of the target region being covered ≥20-fold. We applied different filtering steps to prioritize likely pathogenic variants, including a search for rare (MAF [minor allele frequency] <0.1% in in-house and public databases) homozygous or putatively compound heterozygous variants. From this list (*n* = 26 genes; Supplemental Table 1), we next prioritized the genes listed in the OMIM (phenotype key 3) that have been associated with clinical features of the proband (*n* = 1), leading to the identification of a homozygous frameshift mutation in proprotein convertase subtilisin/kexin type 1 (*PCSK1*; NM_000439.4: c.679del, p.[Val227Leufs*12]) and predicting a prematurely truncated polypeptide missing the last 516 amino acids (68% of the full protein). Sanger sequencing confirmed the variant in a homozygous state in the patient, with his parents being heterozygous carriers. Mutations in *PCSK1* have been reported as a rare cause of early-onset malabsorptive diarrhea and multiple endocrine dysfunction.[6,7] *PCSK1* encodes prohormone convertase 1/3, which is a calcium-dependent serine endoprotease that is essential for peptide hormone processing and activation (eg, proopiomelanocortin, proinsulin, proglucagon, and proghrelin).[8,9] Laboratory workup of the patient further confirmed the diagnosis of prohormone convertase 1/3 deficiency (eg, by detection of massively increased serum proinsulin levels, etc).

Of note, BDS has not been reported in the context of *PCSK1* mutations. However, as demonstrated in Fig 1, the bluish discoloration of diapers is only visible depending on indigo concentrations and urine pH, which explains why this is an inconsistently visible and possibly underrecognized finding. So far, there is no indication from previous studies that *PCSK1* is involved in tryptophan metabolism. However, *PCSK1* is crucial for normal intestinal physiology via the biosynthetic processing of hormone precursors. The small intestine expresses >40 peptide hormones, many of them with unclear functions regarding substrate transport.[10,11] In view of tryptophan metabolism, it is known that this amino acid is absorbed in the small intestine via the sodium-dependent neutral amino acid transporter B(0)AT1 (also known as solute carrier family 6 member 19). Interestingly, it was demonstrated that leptin has an influence on B(0) AT1 transport activity.[12] Of note, the disturbance of the leptin-melanocortin signaling pathway has been discussed in the context of PCSK1 deficiency.[13] However, so far, there is no research evidence available to support a clear association between PCSK1, leptin, and tryptophan metabolism.

Clinical features of the children described in 1964 are partially consistent with *PCSK1* deficiency (diarrhea, inadequate weight gain, and improvement of clinical condition with cortisone therapy) but differ in some aspects (hypercalcemia and nephrocalcinosis). Tryptophan levels in the plasma samples of the child reported here were within normal limits. However, this was also the case in 1 of the patients from the original report. All in all, the study presented here indicates a first link between BDS and *PCSK1* defects. Still, it remains unclear if BDS is a primary feature of *PCSK1* defects (eg, caused by a direct impact of *PCSK1* on tryptophan uptake or metabolism) or if it represents secondary findings among the broad spectrum of *PCSK1*-associated clinical features. Further studies in which researchers measure indigo concentrations in patients with *PCSK1* defects and of infants with malabsorptive diarrhea might help to determine the specificity and prevalence of this phenomenon.

ABBREVIATIONS

BDS: blue diaper syndrome
OMIM: Online Mendelian Inheritance in Man
PCSK1: proprotein convertase subtilisin/kexin type 1
UPLC-ESI-MS/MS: ultra-performance liquid chromatography–electrospray ionization–tandem mass spectrometry

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