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Exome sequencing in a Romanian Bardet-Biedl syndrome cohort revealed an overabundance of causal *BBS12* variants

Sheraz Khan[1,2,3,24], **Ina Ofelia Focșa**[4,5,24], **Magdalena Budișteanu**[6,7,8], **Cristina Stoica**[4,9], **Florina Nedelea**[4,10], **Laurenţiu Bohîlţea**[4], **Lavinia Caba**[11], **Lăcrămioara Butnariu**[11,12], **Monica Pânzaru**[11,12], **Cristina Rusu**[11,12], **Claudia Jurcă**[13,14], **Adela Emandi-Chiriţă**[15,16], **Claudia Bănescu**[17], **Wasim Abbas**[2,3], **Azita Sadeghpour**[18,19], **Shahid Mahmood Baig**[20,21], **Mihaela Bălgrădean**[4,22], **Erica E. Davis**[1,23]

[1]Stanley Manne Children's Research Institute, Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL, USA

[2]Human Molecular Genetics Lab, Health biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE-C) Faisalabad, Pakistan.

[3]Pakistan Institute of Engineering and Applied Sciences (PIEAS), Islamabad, Pakistan.

[4]University of Medicine and Pharmacy "Carol Davila" Bucharest, Romania

[5]Cytogenomic Medical Laboratory, Bucharest, Romania

[6]Psychiatry Research Laboratory, "Prof. Dr. Alexandru Obregia" Clinical Hospital of Psychiatry, Bucharest, Romania

[7]Medical Genetic Laboratory, "Victor Babeș "National Institute of Pathology, Bucharest

[8]Department of Medical Genetics, Faculty of Medicine,"Titu Maiorescu" University, Bucharest, Romania

[9]Department of Pediatrics, Clinical Institute Fundeni, Bucharest, Romania

[10]Genetics Department, Clinical Hospital Filantropia, Bucharest, Romania

[11]Department of Medical Genetics, "Grigore T. Popa" University of Medicine and Pharmacy, Iași, Romania

Correspondence: eridavis@luriechildrens.org.

Authors Contributions

SK performed genetic data analysis/interpretation, variant validation and segregation analysis, manuscript first draft, review and editing of the manuscript; **IOF** collected the cases and performed clinical assessment; **MB, CS, FN, LB, LC, LBu, MP, CR, CJ, AEC, CB, MBa** performed clinical assessment; **AS** assisted with organization of clinical samples and data; **WA and SMB** reviewed and edited the manuscript; **EED** conceptualized and designed the study, reviewed genomic variants, reviewed and edited the manuscript (with input and approval from all authors) and acquired funding. All authors contributed to the manuscript and approved for the final submission.

Ethics Approval/Patient Consent

This study was approved by Institutional Board of the University and Pharmacy Carol Davila Bucharest (approval no. 29700, T.42; Oct 01, 2015) and Ann & Robert H. Lurie Children's Hospital of Chicago (approval no. IRB 2019–3057, August 5, 2019). The families were enrolled in this study under informed consent and all the experiments conformed with the guidelines of the Declaration of Helsinki.

Conflict of Interest

The authors have no competing interests to declare.

[12]Regional Medical Genetics Centre, “Sf. Maria” Children’s Hospital, Iaşi, Romania

[13]Department of Genetics, Faculty of Medicine and Pharmacy, University of Oradea, Romania

[14]Department of Pediatrics, “Dr. Gavril Curteanu” Municipal Clinical Hospital, Oradea, Romania

[15]Emergency Hosp Children Louis Turcanu, Genet Compartment, Timisoara, Romania

[16]Victor Babes Univ Med & Pharm Timisoara, Dept Microscop Morphol Genet, Ctr Genom Med, Timisoara, Romania

[17]“George Emil Palade” University of Medicine, Pharmacy, Sciences and Technology, Târgu Mureş, Romania

[18]Center for Human Disease Modeling, Duke University Medical Center, Durham, NC, USA.

[19]Duke Center for Applied Genomics and Precision Medicine, Duke University, Durham, NC, USA.

[20]Pakistan Science Foundation (PSF), Constitution Avenue, Islamabad, Pakistan

[21]Department of Biological and Biomedical Sciences, Agha Khan University Karachi, Pakistan

[22]Department of Pediatrics and Pediatric Nephrology, Emergency Clinical Hospital for Children “Maria Skłodowska Curie”, Bucharest, Romania

[23]Department of Pediatrics and Department of Cell and Developmental Biology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

[24]Authors contributed equally

Abstract

Bardet-Biedl syndrome (BBS), is an emblematic ciliopathy hallmarked by pleiotropy, phenotype variability, and extensive genetic heterogeneity. BBS is a rare (~1/140,000 to ~1/160,000 in Europe) autosomal recessive pediatric disorder characterized by retinal degeneration, truncal obesity, polydactyly, cognitive impairment, renal dysfunction, and hypogonadism. Twenty-eight genes involved in ciliary structure or function have been implicated in BBS, and explain the molecular basis for ~75–80% of individuals. To investigate the mutational spectrum of BBS in Romania, we ascertained a cohort of twenty-four individuals in twenty-three families. Following informed consent, we performed proband exome sequencing (ES). We detected 17 different putative disease-causing single nucleotide variants or small insertion-deletions and two pathogenic exon disruptive copy number variants in known BBS genes in 17 pedigrees. The most frequently impacted genes were *BBS12* (35%), followed by *BBS4*, *BBS7*, and *BBS10* (9% each) and *BBS1*, *BBS2*, and *BBS5* (4% each). Homozygous *BBS12* p.Arg355* variants were present in seven pedigrees of both Eastern European and Romani origin. Our data show that although the diagnostic rate of BBS in Romania is likely consistent with other worldwide cohorts (74%), we observed a unique distribution of causal BBS genes, including overrepresentation of *BBS12* due to a recurrent nonsense variant, that has implications for regional diagnostics.

Keywords

ciliopathy; retinal dystrophy; polydactyly; urogenital malformations; second-site modifiers; pleiotropy

Introduction

Bardet-Biedl syndrome (BBS [OMIM 209900]), is a rare autosomal recessive pediatric disorder caused by dysfunction of primary cilia[1; 2]. BBS is a multisystem primary ciliopathy characterized by multiple clinical manifestations, most prominently progressive retinal degeneration, postaxial polydactyly, truncal obesity, cognitive impairment, renal dysfunction, and hypogonadism or urogenital malformations. Additional clinical findings of varying frequency that may complicate clinical diagnosis include neurological abnormalities, endocrine and metabolic impairment, cardiovascular defects, brachydactyly/syndactyly, dental anomalies, and gastrointestinal abnormalities[1]. Clinical diagnostic criteria have been proposed as the presence of either four or three major clinical features in combination with at least two minor or secondary features[3]. Certain signs are detectable antenatally; these include polydactyly, kidney anomalies or abdominal distension due to genitourinary abnormalities, and thus raise the suspicion of BBS in early childhood[4; 5]. However, most patients are diagnosed in late childhood or early adulthood, and typically the diagnosis is prompted by the manifestation of retinal dystrophy[3; 6]. The incidence of BBS varies among different populations and is increased in the regions with a high level of endogamy. For instance, in North America and Europe, its prevalence is estimated at around 1:140,000 to 1:160,000 live births[7; 8] while the incidence is elevated in certain isolated populations such as Newfoundland and Kuwait, where the incidence rises to 1:18,000 and 1:13,500, respectively, postulating a founder effect[1; 9; 10].

There are six cardinal features of BBS and multiple infrequent clinical symptoms. Progressive retinal degeneration is a highly penetrant feature evident in the first decade [6; 11; 12] with complete loss of visual acuity by second or third decade of life[13–15]. Obesity usually begins in childhood and becomes obvious during the first 3 years of life[1; 16; 17]. Polydactyly is observed commonly but not always in affected individuals with BBS; it may occur with syndactyly, brachydactyly, and clinodactyly[3; 18; 19]. One of the least understood and disputed features of BBS is cognitive impairment; >62% of patients have been reported to have cognitive difficulties although the severity is highly variable[3; 15; 20]. Functional kidney deficits are variable and often lead to chronic kidney disease (CDK) which is considered a major contributor of morbidity in individuals affected with BBS[21]. Individuals with BBS also display congenital structural abnormalities[22–25]. Hypogenitalism and hypogonadism are reported in nearly all males, while hypoplastic labia minora, vaginal atresia, and septate or imperforate vagina are common in females[9; 26]. Several minor features have also been documented in individuals with BBS, including facial dysmorphism, developmental delay, speech deficit, neurological abnormalities, metabolic and endocrine disturbance, diabetes mellitus, cardiovascular defects and Hirschsprung disease[27; 28]. Assembly and analysis of consistent and longitudinal clinical data from the Clinical Registry Investigating Bardet-Biedl Syndrome (CRIBBS), have refined further the incidence and variability of clinical phenotypes[21; 29; 30].

To date, causal variants in twenty-eight different genes have been linked with BBS (Table S2), all of which are implicated in the structure and/or function of the primary cilium[21; 31; 32]. Pathogenic variants in primary recessive driver loci explain the molecular

basis for 75–80% of cases, suggesting that additional genes remain to be identified[4]. *BBS1* and *BBS10* are major contributors, accounting for nearly half of affected individuals[33–35], although some regional variation in prevalence exists[16]. Causal variants identified in *BBS12* and *ARL6/BBS3* account for ~8% of the clinically diagnosed patients each[36–38], and the remainder of genes account for less than 5%[39]. Copy number variants (CNV) also contribute to the mutational burden of BBS, and exon disruptive CNVs are detectable in up to 18% of clinically assessed cases[32; 40; 41]. BBS is inherited predominantly in an autosomal recessive manner; however, this classical mode of inheritance has been challenged by extensive molecular and functional investigation reporting second-site variation in other BBS genes which could possibly explain phenotypic variability[42–44 45; 46 47–49].

BBS is considered as a model disease to gain insight into the biology of the primary cilium. A subset of disease-associated proteins (BBS1, BBS2, BBS4, BBS5, BBS7, BBS8/TTC8, BBS9, and BBS18/BBIP1) form a multimeric complex known as the BBSome. This multiprotein complex is localized to the base of the cilium and functions as an adaptor for intraflagellar transport (IFT) molecules[50; 51]. Three chaperonin-like proteins (BBS6/MKKS, BBS10, and BBS12) form a complex with chaperonin containing tailless complex polypeptide 1 or tailless complex polypeptide 1 ring complex (CCT/TRiC) family chaperonins and play an essential role in BBSome assembly[52; 53]. Other proteins have roles in BBSome localization and activation (*ARL6/BBS3*)[54], entry into cilia (*BBS17*) or are associated with the BBSome (*BBS14*)[55], whereas the remainder of proteins disrupted in BBS cases are vital to ciliogenesis and ciliary function [56].

Primary cilia function in sensory perception and various signaling pathways including, sonic hedgehog signaling (shh), wingless/integrated (Wnt), notch, salvador warts hippo (SWH), Platelet-derived growth factor receptors (PDGFR), mammalian target of rapamycin (mTOR), and G-protein coupled receptors (GPCR), to regulate developmental processes, tissue plasticity, and organ development[57; 58]. Dysregulation of these signaling pathways has been associated with ciliary dysfunction[59; 60], which results in multiorgan defects. For example, impaired shh signaling may induce digit abnormalities, craniofacial defects, skeletal malformations, and intellectual disability, while dysregulated Wnt signaling is likely a contributor to some renal phenotypes[61]. BBS belongs to a broader clinical group of disorders termed ciliopathies which share a common organellar etiology; phenotypic overlap with other ciliopathies including McKusick-Kauffman syndrome (MKKS), Joubert syndrome (JBTS), Alstrom syndrome (ALMS), and Meckel Gruber syndrome (MKS) have been discussed elsewhere[1; 48; 62].

Here, we report the clinical spectra and genetic analysis of a cohort of 23 families who reside in Romania. Using exome sequencing (ES), we identified 17 different SNVs or small indels and 2 CNVs in 19 families (17 families with a primary recessive locus identified and 2 additional families with heterozygous variants). To analyze CNVs, we characterized breakpoints by long-range PCR and subsequent Sanger sequencing. Additionally, we functionally characterized a rare intronic variant (RefSeq ID: NM_033028.5, *BBS4:* c.332+8T>C) segregating in *trans* with a CNV. Finally, our cohort presents a unique distribution of BBS causal genes, due in part to the recurrence of a *BBS12* nonsense variant (RefSeq ID: NM_152618.3, c.1063C>T) possibly due to a founder effect.

Materials and Methods

Study Participants, Clinical Evaluation, Ethics Approval, and DNA Extraction

The relevant ethics committees of the University of Medicine and Pharmacy Carol Davila Bucharest and Ann & Robert H. Lurie Children's Hospital of Chicago approved the study. We recruited twenty-four affected individuals with BBS and their available family members from twenty-three unrelated families. Clinical evaluation was performed after receiving written informed consent from the legal guardian of pediatric participants, and their adult family members. BBS diagnoses were ascertained by a medical geneticist who identified either four or three major features plus two minor or secondary features according to established criteria[3]. We obtained peripheral blood by venipuncture and extracted genomic DNA from samples using the Purelink® Genomic DNA Extraction kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Individual DM1569 was reported previously[63].

Next-Generation Sequencing, Variant Filtration, and *In Silico* Analyses

To identify the causative genes in our cohort, we performed ES on proband genomic DNA according to an established protocol (LC Sciences, LLC). For exome capture, we used the Agilent SureSelect Human All Exon V6 kit (Agilent Technologies, Inc.) and constructed libraries according to the manufacturer's instructions. Captured libraries were further subjected to paired-end sequencing on an Illumina Novaseq6000 system at Lianchuan Bio, resulting in 150 bp paired-end reads, to a mean depth ranging from 61X-140X across individuals, with 82 % to 96 % of coding regions covered by ≥ 20 reads (Table S1). The detailed methods were adopted for ES as described[63]. Low-quality reads were removed and reads with minimum coverage of ≥ 10 were considered further for analysis. Varsome clinical (10.1) analysis software and Variant Annotation and Filtering Tool (VarAFT), version 2.17–2 (https://varaft.eu/)[64] were used to prioritize SNVs and small indels to retain functional variants with minor allele frequency (MAF) <0.01 in the Genome Aggregation Database (gnomAD v3.1.2) (https://gnomad.broadinstitute.org/), predicted to alter the amino acid sequence and intron-exon junctions in known BBS genes (Table S2). CNVs were identified within the Varsome clinical platform with the ExomeDepth CNV caller (v1.1.11)[65]. Variant pathogenicity was predicted using the following *in silico* tools: MutationTaster2021 (https://www.mutationtaster.org/)[66], Provean (v1.1) (https://www.jcvi.org/research/provean)[67], CADD (v1.6) (https://cadd.gs.washington.edu/)[68], and SIFT (v6.2.1) (https://sift.bii.a-star.edu.sg/)[69] and categorized according to the American College of Medical Genetics (ACMG) classification system[70]. Prioritized variants were inspected visually with the Integrated Genomics Viewer (IGV, Broad Institute). Amino acid conservation was visualized by generating multiple sequence alignments using Clustal Omega (EMBL-EBI, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK) (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Long Range PCR for Variant Phasing and CNV Junction Fragment Analyses

To phase rare variants in families for which neither parental genomic DNA was available (DM1574) or to characterize CNV breakpoints (DM1586, DM1576, and DM1566), we performed long-range PCR, cloning and sequencing. The region of interest was amplified

using the Phusion High-Fidelity PCR Kit (Thermo Fisher Scientific, F5321L) according to the manufacturer's instructions. The *BBS1, BBS4* and *BBS10* amplicons unique to the carrier and not observed in the controls were gel-separated, purified, and subjected to Sanger sequencing.

Sanger Confirmation and Segregation Analysis

For SNV or CNV validation and segregation analysis in genomic DNA from all available family members, we PCR-amplified the targeted regions (primer sequences and PCR conditions are available upon request). PCR products were sequenced bidirectionally using BigDye terminator 3.1 chemistry and an ABI 3730xl DNA analyzer according to the manufacturer's protocols (ThermoFisher Scientific, Inc.). Sequence chromatograms were analyzed by Sequencher® 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). Variant phasing was possible in 9 families with both parental samples available; confirmed in 1 family by long range PCR (see above); and estimated in the remaining families with 1 parental DNA available (n=5) or neither parental DNA available (n=2).

Establishment and Culture of Lymphoblastoid Cell Lines (LCL)

Whole blood samples were collected in BD Vacutainer® ACD A tubes (BD: 0100195). Peripheral blood mononuclear cells (PBMCs) were separated from whole blood, and then approximately 2.5 X 10^6 PBMCs were exposed to Epstein Barr virus to establish LCLs as described[71]. LCLs were cultured in Gibco Roswell Park Memorial Institute (RPMI) supplemented with 10% Heat Inactivated Fetal Bovine Serum and 1% pen-strep (100 IU/ml penicillin and 100 μg/ml streptomycin). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 using standard cell culture protocols.

RNA Isolation and mRNA Splicing Studies

LCLs were harvested for total RNA isolation using Trizol reagent (ThermoFisher Scientific) and subsequently reverse transcribed to cDNA using the QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's guidelines. To investigate impaired splicing, we performed PCR on cDNA obtained from a matched control and an affected individual carrying an intronic variant and an exon 3–4 deletion *in trans* in *BBS4*. The primer pairs used for PCR annealed to exons 1 and 7, upstream and downstream variant sites, respectively. The amplified product was separated on a 2% agarose gel with 1 kb plus ladder (ThermoFisher Scientific), gel slices were purified separately (QIAquick® Gel Extraction Kit, Qiagen) and confirmed by Sanger sequencing.

Results

Clinical manifestations of individuals with BBS

Twenty-four affected individuals with suspected BBS (23 probands and 1 affected sibling) from twenty-three families who reside in Romania (Table 1) were referred from multiple sites. Their self-reported ethnicity includes Eastern European (n=17), Romani (n=5), and Arab (n=1). Among these, our group previously reported one family as a case report[63]. We noted a broad age range at the time of clinical ascertainment (2 months to 43 years) with

both sexes represented in our cohort (9 males; 15 females). The affected individuals were evaluated by multidisciplinary clinical teams and consented for research.

We observed the archetypal BBS features in all affected individuals (Table 1; reported in detail elsewhere)[72]. Retinal degeneration, male hypogonadism, renal anomalies and learning difficulties are the most predominant features. Retinal dystrophy was noted in 17 of 18 individuals for which data are available, 94%. We documented urogenital anomalies in all males (hypogonadism, 9 of 9; 100%). Among females for which data were available, we observed a high incidence of hypoplastic genitalia (12 of 13, 92%), with concomitant congenital vaginal atresia in a minority (2 of 13, 15%). Among the entire cohort, we observed renal anomalies such as hydronephrosis, polycystic kidney, hypoplastic or atrophic kidney and end stage kidney disease in 12 of 12 (100%) individuals for whom data are available. All individuals with cognitive assessment showed intellectual disability, albeit at varying degrees of severity ranging from mild and moderate to severe (19 of 19, 100%). Additionally, we observed obesity in 20 of 24 cases (83%). However, in the other cases (two girls aged two months, one girl and one boy aged ten years) we cannot preclude the possibility that this feature may manifest later in life. Digit anomalies were present in 21 of 24 (92%) cases; these included postaxial polydactyly, syndactyly and brachydactyly and ranged from a single hand or foot to all four extremities.

In addition to classical BBS clinical manifestations, most cases also exhibited secondary or minor features including psychomotor delay (72%), language and speech delay (64%), other neuropsychiatric abnormalities (28%), cardiovascular involvement (28%), metabolic syndrome (28%) and type 2 diabetes (24%) (Table 1).

Genetic analysis of individuals with BBS

To identify the genetic etiology of BBS in our cohort, we performed ES on all affected individuals. We generated 150 bp paired-end reads on an Illumina platform to acquire average target read depth of 106x (61–140x) with 82–96% of bases covered by >20x (Table S1). Bioinformatic filtering identified 17 different causal variants or small indels and two exon disruptive CNVs in known BBS genes in 17 families. Of these, 12 affected individuals harbor causative homozygous SNVs or indels (52%), 3 have compound heterozygous SNVs or small indels (13%), 1 affected individual carries an SNV in *trans* with a CNV (4%), and 1 affected individual harbors a homozygous CNV (4%) in BBS genes (Figures 1, 2, 3; Tables 2 and 3, Table S3 [63]).

Biallelic SNVs or small indels in known BBS genes are predominant in our cohort

A majority of our cohort carry biallelic pathogenic or likely pathogenic SNVs or small indels in established BBS genes. Eleven variants have been reported previously in affected individuals, have high amino acid conservation, and segregated with disease in available family members (Table 2, Table S3, Figure 1, Figures S1 and S2). The majority of previously reported variants were non-recurrent in our cohort, however, we observed a *BBS12* p.Arg355* allele in 7 of 23 probands. Additionally, we identified two hitherto unreported variants in cases: *BBS12* p.Cys464Trpfs*7 and p.Glu561Lysfs*10 leading to a frameshift and predicted premature termination (Table 2, Table S3, Figure 1, Figure S1).

Identification of causal genes disrupted by CNV deletions in known BBS loci

Rare CNV deletions and duplications have been identified in a broad spectrum of human diseases including autism, intellectual disability, and BBS[32; 40; 73; 74]. We found 2 of 17 individuals who carry previously reported recurrent exon disruptive deletions in *BBS1* and *BBS4*[32; 40], respectively, that are inherited under a recessive paradigm.

In family DM1586, we used exome read depth to detect a homozygous 17.7 kb CNV in *BBS1* that deletes exons 1–11 (Figure 2, Table 3). To refine the CNV breakpoint and map to the precise genomic location, we performed long-range PCR and subsequent Sanger sequencing. The CNV junction was located within substrate pairs of *Alu* elements from the same family with 92% sequence identity, suggesting that the deletion was mediated by *Alu-Alu* recombination. This recombination forms an *Alu* hybrid which is the most prominent mechanism underlying the formation of a pathogenic CNV[75–77]. This deletion variant was confirmed heterozygous in both carrier parents, has been reported previously in *trans* with an p.Glu549* SNV in an affected individual with BBS and was deemed as pathogenic[32].

In family DM1566, the affected individual harbored a two-exon deletion in *trans* with an intronic SNV in *BBS4*. This phenomenon was reported previously to contribute to pathology in *BBS1, BBS7*, and *IFT74*[32]. We first characterized the maternally inherited CNV spanning exons 3 and 4. The breakpoint sequencing and analysis of junction fragments showed that the deletion was located within distinct substrate pairs (*AluSc8-AluSx*) with 83% sequence identity; this CNV was reported previously in homozygosity in a BBS case[32]. Next, we evaluated the impact of the paternally inherited heterozygous intronic variant (c.332+8T>C) on mRNA splicing. This change was found once in 250,944 alleles in gnomAD (accessed November 2022). To test whether the variant affects splicing, we established an LCL from whole blood of the affected individual and extracted total RNA. Sequencing of TOPO-cloned RT-PCR product spanning exons 1–7 showed impaired mRNA splicing that results in exon 5 exclusion, resulting in a frameshift p.Arg74Aspfs*7 deletion and putative premature protein termination (Table 2, Figure 3f-g). Sequencing of cloned RT-PCR products also revealed that the maternally inherited CNV results in aberrant mRNA splicing of exons 2 and 5, resulting in an in-frame deletion of 48 amino acids. Together, the segregation data, previous report in BBS cohorts, and our RT-PCR data suggest that these variants are pathogenic.

Identification of secondary contributing variants in BBS genes

We have shown previously that the presence of BBS gene mutational burden is significantly enriched in BBS individuals compared to matched unaffected controls[47]. Among the pedigrees for which we could identify the primary causal locus, three families harbored additional rare heterozygous changes in BBS loci. In family DM1574, with a primary causal *BBS10* locus (with previously reported alleles p.Arg49Trp[37] and Val620Leu[78]), we identified a rare and phylogenetically conserved p.Arg572Gln variant in *IFT172* (Table 2, Table S3; Figures 1, Figure S1 and S2). In family DM1588, with a primary causal *BBS12* locus (homozygous p.Arg355*), we detected a heterozygous p.IIe76Val change in a conserved residue of BBS5 (Table 2, Table S3; Figures 1, Figure S1 and S2). In

family DM1589, also with the same primary causal *BBS12* locus, we identified two rare heterozygous second-site missense variants: *NPHP1*, p.Met544Val and *SCAPER*, p.Arg1098Gln (Table 2, Table S3; Figures 1, Figure S1 and S2).

A subset of BBS cases has unresolved molecular etiology

We did not identify a causal locus for a modest fraction of our cohort (n=6). In two families, we identified heterozygous rare variants in an established BBS gene, each of which were inherited from a single parent. In DM1576, we identified a maternally-inherited *BBS1* allele with two pathogenic changes in *cis*: the recurrent 17.7 kb exon 1–11 disruptive deletion[32] (also identified in DM1586) and the common p.Met390Arg[79] variant (Tables 2 and 3, Table S3; Figures 1 and 2, Figure S1). Additionally, family DM1567 harbors a paternally-inherited putative truncating variant in *BBS7* (p.Arg238Glufs*59). (Table 2; Figure 1, Figure S1). The remaining four families were bereft of rare SNVs, small indels or CNVs in known BBS genes (Figure 1d). Furthermore, unbiased filtering of the exome for any rare, functional biallelic variants yielded no likely causal candidate genes. These families may harbor deep intronic or large structural variants that are intractable to ES.

Discussion

Here, we report the molecular analysis of 24 individuals in 23 families who reside in Romania and fulfill clinical diagnostic criteria for BBS. ES of affected individuals, bioinformatic filtering, and segregation analysis enabled the detection of biallelic likely pathogenic or pathogenic SNVs or CNVs that could potentially inform disease causality in 17 of 23 families. We achieved an overall diagnostic rate of 74%, which is consistent with previous genetic studies on BBS cohorts 70–80%[16; 34; 80; 81]. For one family, we leveraged mRNA profiling from primary LCLs to simultaneously characterize the functional effects of an intronic SNV and an exon disruptive CNV, thus enabling more accurate assessment of variant pathogenicity.

Molecular diagnosis of BBS has been notoriously challenging for three reasons: (1) there are >20 causal genes; (2) a majority of variants are private, and there are few recurrent variants that can be utilized for targeted screening; and (3) a notable portion of contributory BBS alleles are CNVs ranging from small indels to large deletion/complex intragenic duplication events[32]. To potentially circumvent limitations associated with targeted screening, we performed ES, and consistent with previous reports, we identified an allelic series of causal variants that was non-recurrent within our cohort; this includes 13 SNVs or indels. The contribution of CNVs to causality and overall mutational burden has been often under-recognized, and previous studies found that 18% of individuals affected with BBS harbor at least one exon disruptive CNV[32]. Similarly, we detected CNVs in 13% of individuals in our cohort confirming the importance of systematically querying for structural variants.

Notably, we observed three changes in more than one family within our cohort: *BBS1* exon 1–11 CNV deletion; *BBS7* p.Arg238Glnfs*59; and *BBS12* p.Arg355*. The latter SNV was detected in a surprising fraction of families (7 of 23), with no correlation to self-reported ethnicity (4 Romani and 3 Eastern European). Accordingly, p.Arg355* has been reported in BBS cases of Romani origin[37] but is present in gnomAD in both Latino/Admixed American

and non-Finnish European populations. Additionally, while all p.Arg355* alleles were found in homozygosity, only 1 of 7 pedigrees self-reported as consanguineous (DM1585). However, given the lack of parental DNAs for a subset of these families, we cannot exclude the possibility of uniparental disomy. Further studies will be required to determine whether p.Arg355* is a founder allele or mutational hotspot. Further, we note with interest that among p.Arg355* homozygotes, 3 of 7 individuals with recorded cognitive testing have severe intellectual disability, however our cohort is too small to determine whether there is a significant genotype-phenotype correlation.

In our cohort, the frequently impacted genes were *BBS12* (35%), followed by *BBS4, BBS7*, and *BBS10* (9%; 2 families each), whereas *BBS1, BBS2*, and *BBS5* were detected in (4%; 1 family each) of the cases (Figure 4). Although our cohort size is small, the relative causal gene contribution differs from what has been reported from BBS population studies comprised of individuals of northern European descent. *BBS1* and *BBS10* are reported to be predominant drivers of BBS, largely due to the recurrent p.Met390Arg and p.Cys91Leufs*5 variants, respectively[33; 36; 82; 83]. However, there were a paucity of these two common changes in our Romanian cohort (DM1576, *BBS1* p.Met390Arg in *cis* with exon 1–11 CNV deletion; *BBS10* p.Cys91Leufs*5 was not detected). Instead, *BBS12* p.Arg355* was overabundant in our cohort and contributed to *BBS12* emerging as the most common causal gene. Still, *BBS12* is among the major contributors to BBS accounting for 8–11% in most reported cohorts[36; 37], including a recent study of 99 affected individuals for which *BBS12* was causal in 14% of cases[43].

A minority of affected individuals in our cohort harbor secondary variants outside of their primary causal BBS locus (3 of 17, Table 2), but these cases have no apparent distinguishing clinical features or increased severity. Although the rarity of BBS and underlying genetic heterogeneity limit conclusive statements about commonly observed gene pairings, some genes appear to be more frequently implicated in oligogenic phenomena. In a recent study, *BBS1, BBS4, BBS2, CFAP418/BBS21*, and *BBS12* were reported as common driver genes involved in oligogenic phenomena[43]. Accordingly, we detected a third allele in two families with primary causal variants in *BBS12*. Further, we have speculated previously that gene pairings involving proteins known to function in different molecular complexes drive more potent phenotypes (e.g. chaperonin-BBSome or chaperonin-IFT pairings) than inter-module pairings (e.g. BBSome-BBSome or chaperonin-chaperonin)[47]. Consistent with this notion, all three families with secondary variants involve gene combinations encoding different complexes. However, we are cautious about drawing formal conclusions given the small sample size of our cohort.

Finally, a small fraction of families in our cohort remains molecularly undiagnosed (6 of 23; 26%). We detected heterozygous rare variants in two families, but further investigation will be required to determine whether they are primary causal alleles with the second variant undetected with our current ES methodology, or they are secondary contributor variants. The remaining families harbored neither rare heterozygous variants in known BBS genes, nor rare variants in hitherto unreported ciliopathy genes elsewhere in the exome. We speculate that they might harbor regulatory variants in non-coding regions or large deletions that are

intractable to ES. The eventual transition to the whole genome sequencing combined with RNA sequencing will likely overcome this challenge[84].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

Variants identified have been deposited in ClinVar: https://www.ncbi.nlm.nih.gov/clinvar/

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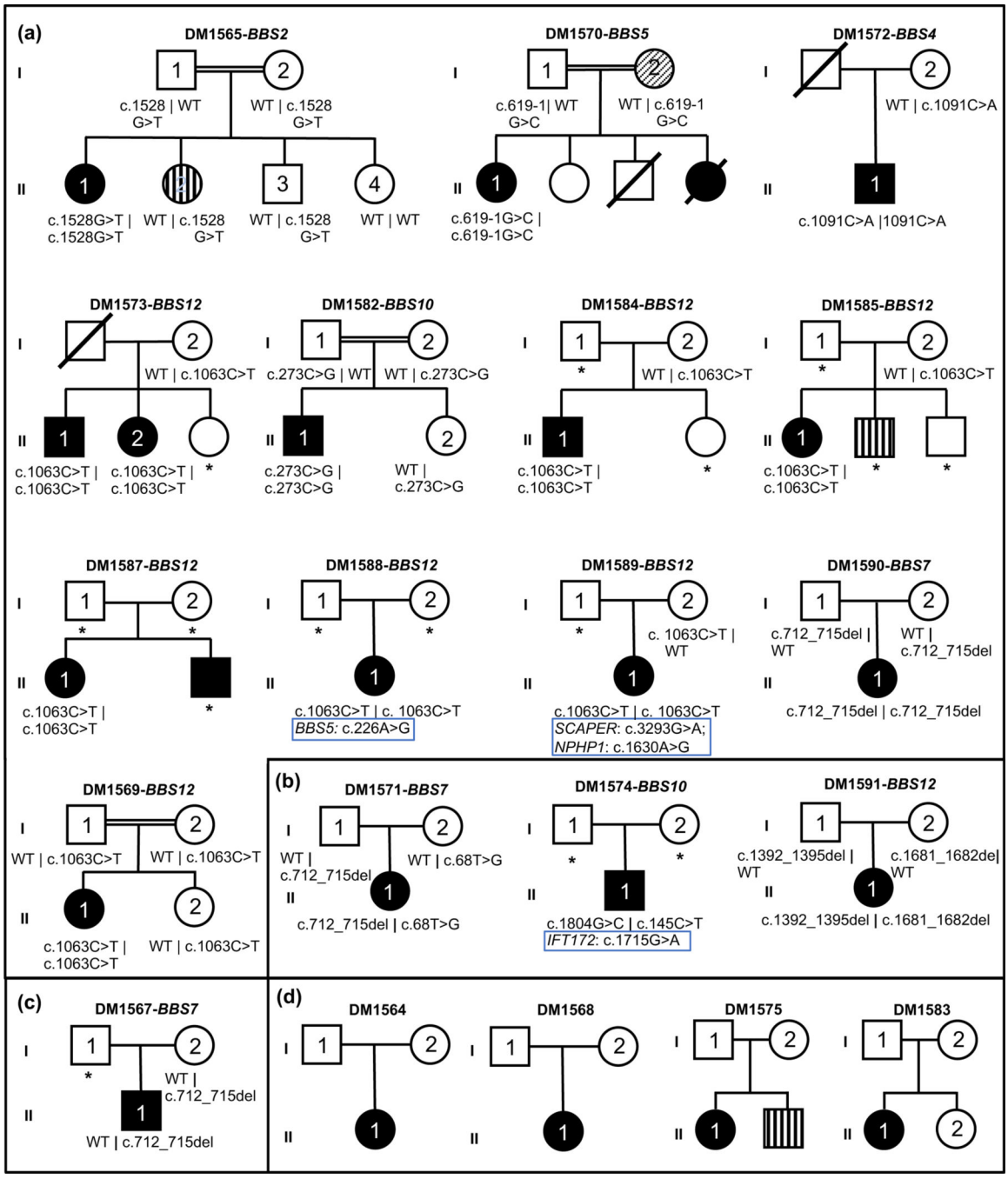

Figure 1. Sixteen pedigrees harboring single nucleotide variants or small indels in BBS genes and four unresolved pedigrees.

Pedigrees and genotyping data of BBS gene variants (SNVs or small indels). Causal gene and family identifier (denoted by DM number) are mentioned on top of each pedigree. Symbols indicate the following: square, male; circle, female; unfilled, unaffected individual; black filled, individual affected with BBS; vertical striped shape, individual affected with neurodevelopmental disorder; diagonal striped shape, individual affected with polycystic kidney disease and end-stage kidney disease; diagonal line, deceased individual; double horizontal lines, consanguinity; WT, wild type; asterisk (*), no DNA available. (a)

families carrying homozygous variants; see DM1569 in [63]; (b) families with compound heterozygous changes; (c) families with heterozygous variants; (d) unresolved families. Wherever applicable modifiers or secondary loci are listed under the primary causal alleles and highlighted by blue rectangles.

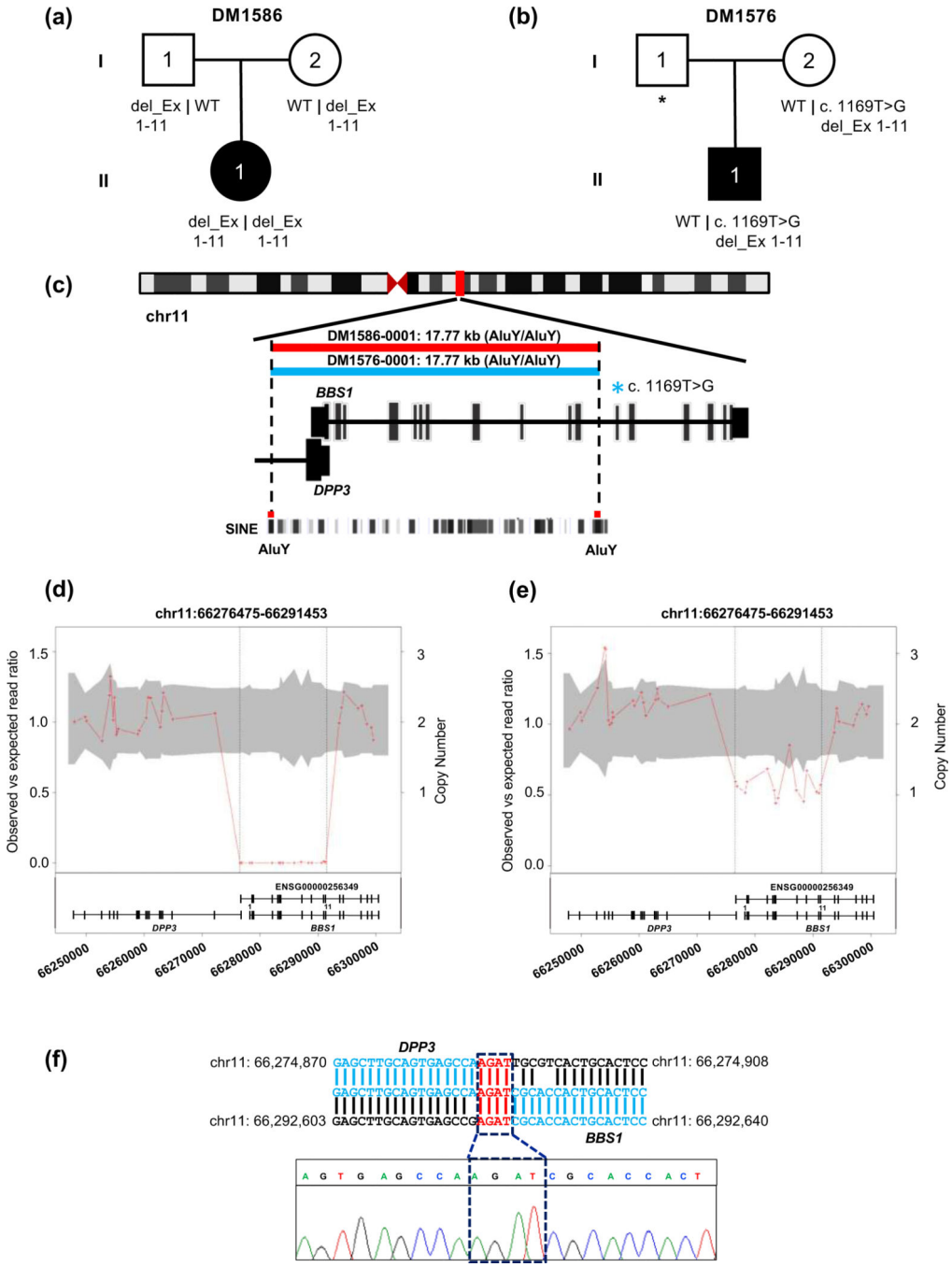

Figure 2. Characterization of a 17.77 kb CNV deletion in *BBS1* in family DM1586 and family DM1576.

(a-b) Pedigrees and segregation of *BBS1* exon disruptive deletion. (c) Schematic representation of human chromosome 11 and location of *BBS1* CNV deletion is indicated with vertical red bar; enlarged view shows schematic of *BBS1* transcript and location of *AluY-AluY* repeats elements. Short interspersed nuclear elements (SINE) (d-e) CNV plot showing homozygous and heterozygous *BBS1* deletion, the gray area marks 95% confidence interval and the vertical black dotted lines indicate the location of the CNV; bottom, schematic of *BBS1* locus: vertical bars, exons; horizontal line, intronic region;

coordinates on chromosome 11 (hg19) are shown. (f) *BBS1* breakpoint junction and sequence chromatograms amplified from genomic DNA of DM1586-0001 (II-1); a 4 bp microhomology region is present at the junction of *DPP3* and *BBS1*, highlighted in red.

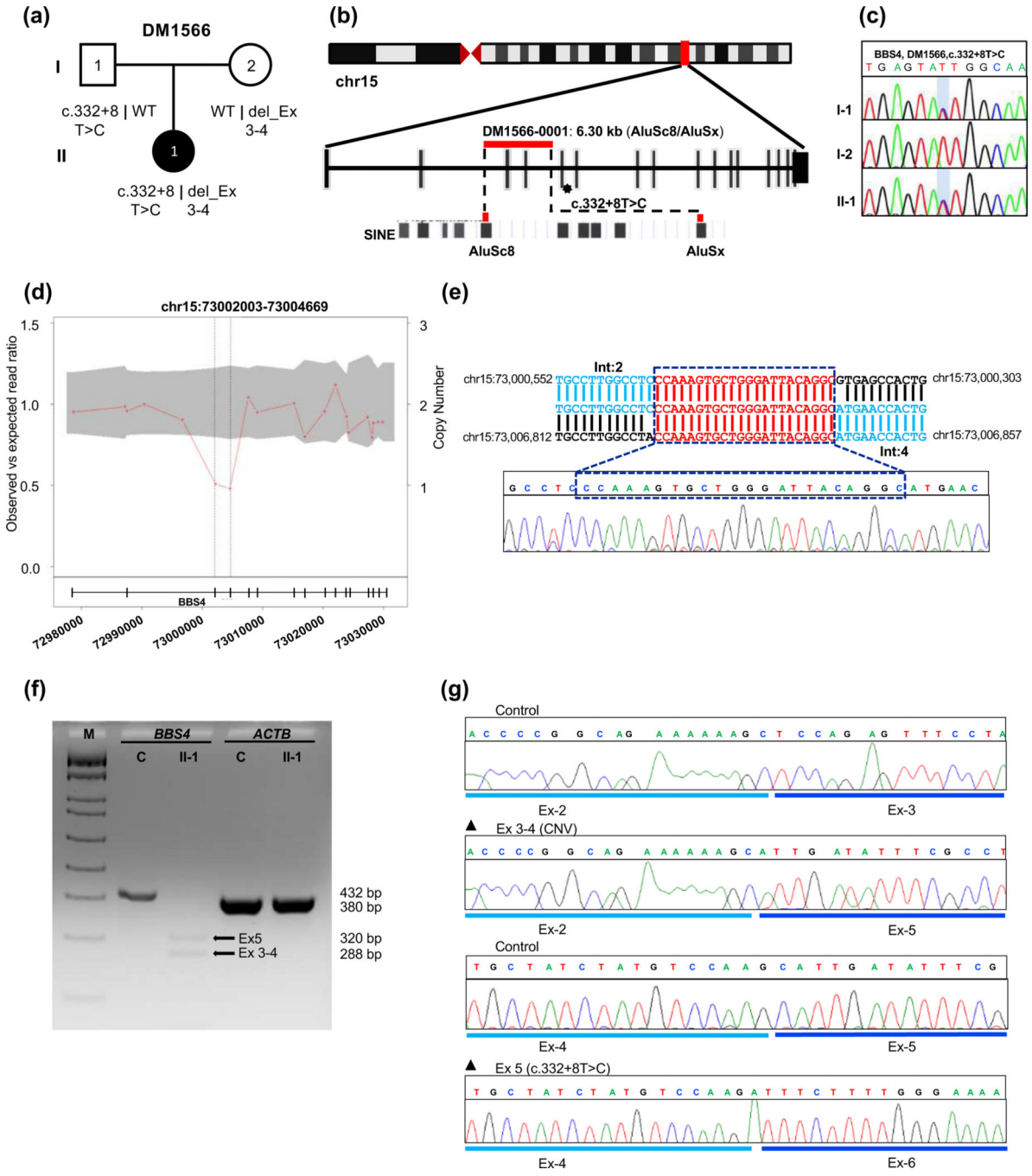

Figure 3. Genetic analysis of DM1566 harboring a biallelic two-exon deletion *in trans* with a pathogenic splicing variant in *BBS4*.

(a) Pedigree and segregation analysis of *BBS4* variants in DM1566. (b) Schematic representation of human chromosome 15 showing the location of *BBS4* with vertical red bar; enlarged view of CNV and SNV bearing region of BBS4 correspond to red horizontal bar and asterisk respectively; AluSc8 and AluSx are indicated at bottom. (c) Sequence chromatogram of paternally-inherited c.332+8T>C change, the variant position is shaded with a light blue vertical bar. (d) CNV plot showing a pathogenic heterozygous two-exon deletions in *BBS4*, the gray area marks a 95% confidence interval. Black vertical dotted

lines indicate the position of the CNV. Bottom, schematic of the *BBS4* locus; vertical bars, exons; horizontal line, intronic region; numbers indicate genomic coordinates on chromosome 15 (hg19). (e) *BBS4* breakpoint junction sequence in genomic DNA; reference location highlighted in blue and a 22 bp microhomology region present at the junction is shown in red; Int, intron. (f) RT-PCR results show impaired splicing in proband cDNA; 2% agarose gel showing *BBS4* amplification products in unaffected control (C, 432 bp expected wild type product) and affected individual II-1 (320 bp band showing exon-5 skipping due to splicing variant and 288 bp band indicating exon 3-4 deletion due to CNV); *ACTB* (380 bp) was amplified to ensure RNA integrity for both control and proband; M, DNA marker; Ex, exon. (g) Sequence chromatograms of RT-PCR products from unaffected control (top) and DM1566 proband show aberrant mRNA splicing outcomes from maternally inherited exon 3-4 deletion (middle) and paternally inherited exon 5 skipping (bottom).

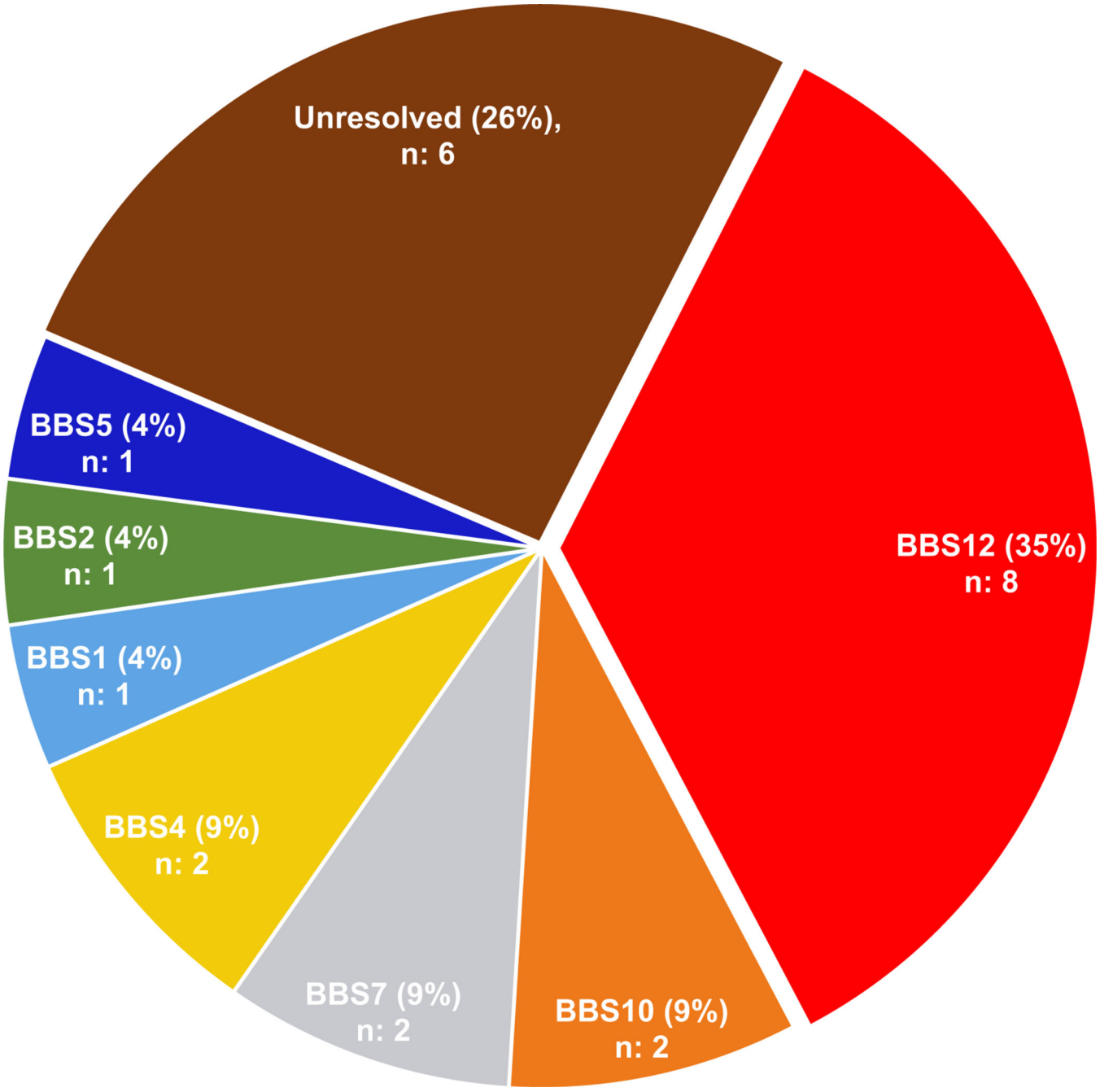

Figure 4: Distribution of BBS gene contribution to the molecular etiology of our cohort.
Pie chart shows the genetic architecture of primary causal BBS genes in a cohort of 23 unrelated families.

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Table 1.

Summary of main BBS related clinical manifestations and overlapping features

Family ID	Sex	Age	BMI (kg/m2)	Main clinical features					Minor or secondary clinical features												Others
				RP	PD	Ob	HG	RD	ID	DD	BD	SYN	MY	AST	Str	HAP	SLI	MC	BC	PF	
DM1564	F	10 yrs	22.1	+	+	ND	+	ND	ND	ND	−	−	+	−	+	+	−	−	−	−	LD
DM1565	F	29 yrs	36.2	+	+	+	+	+	ND	ND	+	−	+	−	−	−	−	−	+	+	DM, HCL, HH, PCOS SS,HT
DM1566	M	15 yrs	32.4	+	+	+	+	+	mild	+	+	−	+	+	+	−	−	−	+	+	CTEV, ROM, HTG, XN, PP
DM1567	M	5 yrs	17.4	ND	+	ND	+	ND	severe	+	−	−	−	−	−	−	−	−	+	−	Atx, BA, AT, VI, XN, MG, CD, Sn
DM1568	F	6 yrs	24.7	ND	+	+	+	ND	moderate	+	+	+	−	−	+	−	+	−	−	+	S, SVT, HD, BA, AT
DM1569	F	6 yrs	31.7	+	+	+	+	ND	severe	−	+	+	−	−	−	−	−	+	−	−	PMA, BA, XN
DM1570	F	39 yrs	52.7	+	+	+	+	+	moderate	ND	−	+	+	+	+	−	−	−	−	−	HF, HT, DM, NYS, PC
DM1571	F	42 yrs	36.4	+	+	+	+	+	ND	+	−	−	+	−	+	−	−	−	+	−	DBA, ONA, HT, SS, PCOS
DM1572	M	18 yrs	35.3	−	+	+	+	ND	moderate	+	−	+	−	−	−	−	−	−	−	−	HH, ASD, RTG, Sn, BA
DM1573–1	M	21 yrs	37	+	−	+	+	ND	moderate	+	+	+	−	−	−	−	−	+	+	−	T2DM, FL, HT
DM1573–2	F	16 yrs	54.7	+	+	+	ND	ND	severe	+	+	−	−	−	−	−	−	+	−	+	BA, HT, T2DM, HTG, HCM, FL
DM1574	M	20 yrs	34.9	+	+	+	+	+	moderate	+	−	−	+	+	−	−	+	−	−	−	HT, HCL, HTG, FL
DM1575	F	8 yrs	30.4	+	+	+	−	ND	severe	+	−	−	−	−	−	−	+	+	−	+	BA, FL, HM, VSD, Atx
DM1576	M	16 yrs	31.9	+	−	+	+	ND	mild	ND	−	−	−	−	−	+	−	+	−	+	FL, SL
DM1582	M	33 yrs	34.7	+	−	+	+	+	mild	+	−	−	−	−	−	−	−	−	−	−	LD, HCL, FL, CTEV
DM1583	F	15 yrs	27	+	+	+	+	+	mild	+	−	+	+	+	+	+	+	−	+	+	HT, ASD
DM1584	M	7 yrs	24.2	+	+	+	+	ND	mild	−	+	−	−	−	−	−	+	−	−	+	HM, HTG
DM1585	F	7 yrs	22.6	+	+	+	+	ND	severe	+	+	−	−	−	−	−	−	−	+	+	Cat, VI
DM1586	F	13 yrs	33.4	+	+	+	ND	+	moderate	+	−	+	−	−	−	−	−	+	−	+	−
DM1587	F	2 mo	−	ND	+	+	+	+	ND	−	−	−	−	−	−	+	−	+	+	+	IH
DM1588	F	13 yrs	28.1	+	+	+	+	+	moderate	−	−	−	−	−	−	+	−	−	−	−	AN, VI, PMD
DM1589	M	3 yrs	29.5	ND	+	ND	+	ND	moderate	−	−	−	−	−	−	−	−	−	−	−	VI, PMD

Family ID	Sex	Age	BMI (kg/m2)	Main clinical features						Minor or secondary clinical features											Others
				RP	PD	Ob	HG	RD	ID	DD	BD	SYN	MY	AST	Str	HAP	SLI	MC	BC	PF	
DM1590	F	5 yrs	31,2	ND	+	+	+	+	mild	−	−	−	−	−	−	+	+	+	−	+	CVA, PF, PMA, HTG, HCL
DM1591	F	2 mo	−	ND	+	ND	+	+	ND	−	−	−	−	−	−	+	−	−	−	+	CVA, UTIs, RRIs

Phenotypes are indicated as present (+) and absent (−). ND indicates no data available, or subject was not evaluated. ASD, atrial septal defect; AST, astigmatism; AT, autistic trait; Atx, ataxic gait; AN, acantosis nigricans; BA, behavior abnormalities; BC, brachycephaly; BD, brachydactyly; BMI, body mass index; Cat, cataract; CD, clinodactyly; CTEV, congenital talipes equinovarus; CVA, congenital vaginal atresia; DBA, delayed bone age; DD, developmental delay; DM, diabetes mellitus; T2DM, diabetes mellitus type 2; FL, fatty liver; F, female; HAP, high arch palate; HCL, hypercholesterolemia; HD, hypodontia; HF, hepatic fibrosis; HG, hypogonadism; HH, hepatic hemangioma; HM, hepatomegaly; HT, hypothyroidism; HTG, hypertriglyceridemia; ID, intellectual disability; IH, imperforate hymen; HCM, hypertrophic cardiomyopathy; LD, learning difficulties; MC, macrocephaly; M, male; MG, micrognathia; mo, months; MY, myopia; NYS, nystagmus; Ob, obesity; ONA, optic nerve atrophy; PC, presbycusis; PCOS, polycystic ovaries; PD, polydactyly; PF, down slated palpebral fissures; PMA, psychomotor hyperactivity/agitation; PMD, psychomotor delay; PP, puberphonia; RD, renal disorder; ROM, recurrent otitis media; RP, retinitis pigmentosa; RRIs, recurrent respiratory infections; RTG, retrognathia; SL, skin lesions; SLI, speech and language impairment; Sn, synophrys; SS, short stature; Str, strabismus; SYN, syndactyly; UTIs, recurrent urinary tract infections; VI, vision impairment; VSD, ventricular septal defect; XN, night blindness; yrs, years.

Table 2.

SNVs and small indels identified in known BBS genes in 18 affected individuals with BBS (primary causal alleles or secondary alleles)

Family ID	Ethnic Origin	Cons.	BBS gene	Transcript ID	Nucleotide change	Amino acid change	ACMG Class	dbSNP ID	Ref	gnomAD*	
										Allele counts (Hom/Het/Wt)	Frequency
DM1565	Arab	YES	*BBS2*	NM_031885.5	c.1528G>T (H)	p.Val510Phe	LP	–	85	–	–
DM1566	E. Eur	NO	*BBS4*	NM_033028.5	c.332+8T>C (h)	p.Ala74Aspfs*7	P	rs1456405256	–	0/01/250944	3.98e-6
DM1567	E. Eur	NO	*BBS7*	NM_176824.3	c.712_715del (h)	p.Arg238Glufs*59	P	rs760165634	86	0/13/250940	5.18e-5
DM1569	Rom	NO	*BBS12*	NM_152618.3	c.1063C>T (H)	p.Arg355*	P	rs121918327	37	0/07/251322	2.79e-5
DM1570	E. Eur	NO	*BBS5*	NM_152384.3	c.619–1G>C (H)	–	P	rs753234582	78	0/04/250308	1.60e-5
DM1571	E. Eur	NO	*BBS7* *BBS7*	NM_176824.3	c.68T>G (h) c.712_715del (h)	p.Leu23Arg p.Arg238Glufs*59	LP P	rs1727380420 rs760165634	** 86	−0/13/250940	−5.18e-5
DM1572	E. Eur	NO	*BBS4*	NM_033028.5	c.1091C>A (H)	p.Ala364Glu	LP	rs28938468	87	–	–
DM1573	Rom	NO	*BBS12*	NM_152618.3	c.1063C>T (H)	p.Arg355*	P	rs121918327	37	0/07/251322	2.79e-5
DM1574	E. Eur	NO	*BBS10* *BBS10*	NM_024685.4	c.145C>T (h) c.1804G>C (h)	p.Arg49Trp p.Val602Leu	LP LP	rs768933093 rs778431173	88 88	0/15/279274 0/01/248576	5.37e-5 4.02e-6
			IFT172	NM_015662.3	c.1715G>A (h)	p.Arg572Gln	VUS	rs764302265	–	0/13/282810	4.60e-5
DM1576	E. Eur	NO	*BBS1*	NM_024649.5	c.1169T>G (h)	p.Met390Arg	P	rs113624356	79	0/444/282790	1.57e-3
DM1582	E. Eur	YES	*BBS10*	NM_024685.4	c.273C>G (H)	p.Cys91Trp	LP	rs148374859	89	0/07/248572	2.82e-5
DM1584	E. Eur	NO	*BBS12*	NM_152618.3	c.1063C>T (H)	p.Arg355*	P	rs121918327	37	0/07/251322	2.79e-5
DM1585	E. Eur	YES	*BBS12*	NM_152618.3	c.1063C>T (H)	p.Arg355*	P	rs121918327	37	0/07/251322	2.79e-5
DM1587	Rom	NO	*BBS12*	NM_152618.3	c.1063C>T (H)	p.Arg355*	P	rs121918327	37	0/07/251322	2.79e-5
DM1588	Rom	NO	*BBS12*	NM_152618.3	c.1063C>T (H)	p.Arg355*	P	rs121918327	37	0/07/251322	2.79e-5
			BBS5	NM_152384.3	c.226A>G (h)	p.Ile76Val	VUS	rs763696357	–	0/05/250968	1.99e-5
DM1589	E. Eur	NO	*BBS12*	NM_152618.3	c.1063C>T (H)	p.Arg355*	P	rs121918327	37	0/07/251322	2.79e-5
			SCAPER	NM_020843.4	c.3293G>A (h)	p.Arg1098Gln	VUS	rs771454836	–	0/05/248920	2.01e-5
			NPHP1	NM_000272.5	c.1630A>G (h)	p.Met544Val	VUS	rs373953762	–	0/01/251420	3.98e-6
DM1590	E. Eur	NO	*BBS7*	NM_176824.3	c.712_715del (H)	p.Arg238Glufs*59	P	rs760165634	86	0/13/250940	5.18e-5

Family ID	Ethnic Origin	Cons.	BBS gene	Transcript ID	Nucleotide change	Amino acid change	ACMG Class	dbSNP ID	Ref	gnomAD*	
										Allele counts (Hom/Het/Wt)	Frequency
DM1591	E. Eur	NO	*BBS12* *BBS12*	NM_152618.3	c.1392_1395del (h) c.1682_1682del (h)	p.Cys464Trpfs*7 p.Glu561Lysfs*10	P P	– –	– –	– –	– –

*The Genome Aggregation Database v2.1.1 (https://gnomad.broadinstitute.org/); all populations;

**ClinVar accession VCV000940124.1. Abbreviations: Hom, homozygous; Het, heterozygous; WT, wild type reference. Abbreviations: Cons., consanguinity; E. Eur, European; H, homozygous; h, heterozygous; LP, likely pathogenic; P, pathogenic; Rom, Romani, VUS, variant of uncertain significance. Modifiers or secondary alleles are listed under primary causal loci wherever applicable.

Table 3.

Breakpoint features of CNVs identified in 3-affected individuals

Family ID	Origin	Cons.	Locus	hg19 CNV Coordinates	Result	Size (bp)	Rearrangement type	Ref	Breakpoint features	% Identity	ACMG Class
DM1586	E. Eur	NO	*BBS1*	chr11:66,274,870-66,292,647	Hom del:	17,777	Simple non-recurrent	[32]	*AluY-AluY*	92%	P
					Exon1_11(H)						
DM1576	E. Eur	NO	*BBS1*	chr11:66,274,870-66,292,647	Het del:	17,777	Simple non-recurrent	[32]	*AluY-AluY*	92%	P
					Exon1_11(h)						
DM1566	E. Eur	NO	*BBS4*	chr15:73,000,552-73,006,857	Het del:	6,305	Simple non-recurrent	[32]	*AluSc8-AluSx*	83%	P
					Exon3_4(h)						

Abbreviations are as follows: CNV, copy number variation; Cons., consanguinity; del, deletion; H, homozygous; h, heterozygous; E. Eur, Eastern European