Diplomarbeit

Molecular characterization of two consanguineous Pakistani families with Bardet-Biedl syndrome

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Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am 22.06.2020 Sophie Therese Bierbaumer eh

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Ich möchte mich an dieser Stelle bei allen Menschen bedanken, die mich beim Verfassen dieser Diplomarbeit unterstützt haben. Mein besonderer Dank gilt Christian Windpassinger, der mir ermöglicht hat, dieses Thema zu bearbeiten und mich mit Geduld und Engagement bei dieser Arbeit begleitet hat.

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Zusammenfassung

Einleitung: Das Bardet-Biedl Syndrom ist eine autosomal rezessive Erbkrankheit, die durch Retinadystrophie, Polydaktylie, Übergewicht, Intelligenzminderung, Hypogonadismus und Nierenfunktionsstörungen charakterisiert ist. Sie gehört zur Gruppe der Ziliopathien, da Defekte im primären Zilium zur Ausbildung der Erkrankung führen. 24 Gene sind bisher als krankheitsverursachend bekannt.

Methoden: Zwei konsanguine pakistanische Familien wurden molekulargenetisch untersucht, um die kankheitsverursachenden genetischen Veränderungen zu bestimmen. Zuerst wurden die beiden häufigsten mutierten Gene (*BBS1* und *BBS10*) mittels Mikrosatelliten auf Homozygotie untersucht. Beide betroffenen Individuen wurden dann mittels Whole Exome Sequencing sequenziert und die Segregation der krankheitsverursachenden Variante innerhalb der Familie durch Sanger Sequenzierung bestätigt.

Resultate: Bei beiden Betroffenen wurde eine bereits beschriebene homozygote Deletion c.299delC in *BBS9* identifiziert, die zur Verschiebung des Leserasters und zu einem vorzeitigen Abbruch der Proteinbiosynthese führt (p.Ser100Leufs*24). Da der Genomabschnitt, der diese Veränderung trug, bei beiden Individuen ident war, wurde die weiter zurückreichende Abstammung beider Familien erkundet und Hinweise auf eine mögliche Verwandtschaft mit dem Stamm der Khaisoori gefunden.

Diskussion: Das Bardet-Biedl Syndrome ist eine phänotypisch und genotypisch sehr variable Erkrankung. Die beiden betroffenen Individuen dieser Studie zeigen trotz identem Genotyp eine unterschiedlich schwere Ausprägung der Erkrankung, insbesondere in Hinsicht auf den Grad der Intelligenzminderung. Diese Studie erweitert den Bestand der klinischen und genetischen Beschreibung des Bardet-Biedl Syndroms und beschreibt eine mögliche „Gründermutation“ im Khaisoori Stamm, die bei der molekulargenetischen Diagnostik betroffener Individuen zu berücksichtigen ist.

Abstract

Introduction: Bardet-Biedl syndrome is an autosomal-recessive disorder characterized by retinal dystrophy, polydactyly, central obesity, mental retardation, hypogonadism and renal dysfunction. It belongs to the group of ciliopathies, as defects in the primary cilium lead to disease manifestation. To date, 24 genes are known to cause the disorder.

Methods: Two consanguineous Pakistani families were molecularly characterized to identify the disease causing variant. The most frequently mutated genes in Bardet-Biedl Syndrome (*BBS1* and *BBS10*) were investigated for homozygosity using microsatellite markers. Both affected individuals were then sequenced by whole exome sequencing, and segregation of the candidate variant within the families was confirmed by Sanger sequencing.

Results: Whole exome sequencing identified a recently reported homozygous deletion in *BBS9* in both individuals. This deletion leads to a shift in the reading frame and to the premature termination of the protein synthesis. As the segment harboring the variant was identical in both individuals, ancestry of the families was explored for more ancient relation and a possible relation with the Khaisoori tribe was established for both.

Discussion: Bardet-Biedl syndrome is a genotypically and phenotypically divers disorder. Both individuals of this study exhibit a variable severity of the phenotype, despite harboring the same genotype. This is most dramatically seen in the different severity of intellectual disability. This report extends the clinical and genetic description of Bardet-Biedl syndrome and describes the possibility of a founder mutation in the Khaisoori tribe, which should be taken into account for the molecular diagnosis of affected individuals.

Angaben von bereits erfolgten Veröffentlichungen

Diese Diplomarbeit entstand im Rahmen einer Studie, die im Journal „Molecular Genetics & Genomic Medicine“ des John Wiley & Sons Verlag erschienen ist.

*Muzammal M, Zubair M, Bierbaumer S, Blatterer J, Graf R, Gul A, et al. Exome sequence analysis in consanguineous Pakistani families inheriting Bardet-Biedle syndrome determined founder effect of mutation c.299delC (p.Ser100Leufs*24) in BBS9 gene. Mol Genet Genomic Med. 2019 Aug;7(8).*

Die Rekrutierung der teilnehmenden Familien, die klinische Untersuchung sowie die DNA-Isolierung fanden unter der Leitung von Muzammil Ahmad Khan statt. Die klinischen Daten der Patienten für diese Diplomarbeit wurden aus der Publikation entnommen.

Inhaltsverzeichnis

Abbreviations

ALMS: Alström syndrome

ARC: arcuate nucleus

BAM: Binary Alignment Map

BBS: Bardot-Biedl syndrome

BMI: Body mass index

Bp: base pair

CDK: chronic kidney disease

dbSNP: Single Nucleotide Polymorphism Database

ddNTP: didesoxinucleotide

dNTP: desoxinucleotide

DNA: desoxiribonucleic acid

GnomAD: Genome Aggregation Database

HGMD: Human Gene Mutation Database

HGNC: HUGA Gene Nomenclature Committee

IBD: identical by descent

IFT: intraflagellar transport

IQ: intelligence quotient

JATD: Jeune's asphyxiating dystrophy

JBTS: Joubert syndrome

LCA: Leber congenital amaurosis

LMS: Laurence-Moon syndrome

MKKS: McKusick-Kaufman syndrome

MKS: Meckel-Gruber syndrome

NGS: next generation sequencing

NPHP: Nephronophthisis

NPY: neuropeptide Y

PCR: polymerase chain reaction

PolyPhen2: Polymorphism Phenotyping v2

POMC: proopiomelanocortin

PTH: Parathyroid hormone

PTHB1: Parathyroid hormone responsive protein B1

PYY: peptide tyrosine tyrosine

RCSB PDB: Protein Databank of the Research Collaboratory for Structural Bioinformatics

Rcf: relative centrifugal force

ROH: run on homozygosity

RP: Retinitis pigments

SIFT: Sorting Intolerant from Tolerant

Smo: Smoothened

SNP: single nucleotide polymorphisms

STR: Short tandem repeats

Taq: Thermus aquaticus (thermophilic bacterium)

TBE-Puffer: Tris-Borat EDTA Puffer

Tris: Trishydroxymethylaminomethan hydrochloride

VCF: Variant Call Format

WES: whole exome sequencing

WGS: whole genome sequencing

Wnt: wingless

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1 Introduction

Bardet-Biedl syndrome (BBS, MIM 209900) is an autosomal recessive disorder affecting the primary cilium. The main features include retinal dystrophy, polydactyly, central obesity, mental retardation, hypogonadism and renal dysfunction. Other features, such as diabetes mellitus, congenital heart disease or development delay can be present. The phenotype develops during the first decade of life, but exhibits a great inter- and intrafamilial variety.[1]

The symptom-complex was first described by Georges Bardet in 1920, when he reported about a girl with truncal obesity, retinitis pigmentosa and hexadactyly of one foot.[2] Another case was presented by Arthur Biedl in 1922 in a lecture in Prague, reporting about a pair of siblings with obesity, retinitis pigmentosa, polydactyly, anal atresia as well as mental retardation.[3] BBS shares features with the much rarer Laurence-Moon syndrome (LMS, MIM 245800), which is characterized by mental retardation, retinal dystrophy, obesity and spastic paraparesis. While the term Laurence-Moon-Bardet-Biedl syndrome has been used without distinction for both syndromes, they are now being considered two distinct disorders with the absence of polydactyly characteristic for LMS.[4]

1.1 Clinical overview

1.1.1 Diagnostic criteria

Today, the clinical diagnosis of BBS is made based on the diagnostic criteria proposed by Beales et al. in 1999: Four primary or three primary and two secondary features are required for the diagnosis. An overview of these features can be found in Table 1.

Primary features	Selection of most common **secondary features**
Rod-cone dystrophy	Speech delay
Polydactyly	Developmental delay
Obesity	Diabetes mellitus
Learning disabilities	Dental anomalies
Hypogonadism in males	Asthma
Renal anomalies	Congenital heart disease
	Brachydactyly/syndactyly
	Ataxia/poor coordination
	Anosmia/hyposmia
	Hearing loss
	Behavioural problems

Table 1: Primary and most common secondary features of BBS. Adapted from Beales et al. (1999)[5] and Forsythe et al. (2013)[1].

1.1.2 Primary features

Polydactyly and limb defects: Beales et al. reported polydactyly in 69% of cases, most commonly postaxial (on the lateral border of the hand or foot). One to four of the limbs were affected. Other skeletal malformations included short, broad and stubby toes, abnormally curved digits and webbed or joined digits (syndactyly). Polydactyly is often the only symptom visible at birth.[5]

Retinal dystrophy and ocular features: Beales et al. reported rod-cone dystrophy in 93% of patients. Unaffected were all younger than eight years old. The first signs of visual troubles were noted at the mean age of 8.5 years and legal blindness was reported at the mean age of 15.5 with a seven year progression from diagnosis to blindness.[5]

Rod-cone dystrophy is the most common presentation of retinal dystrophy in BBS. It is characterized by a primary loss of rod-cells, followed by a secondary loss of cone-cells. In accordance, loss of night vision is usually the first sign noticed by patients themselves or their parents. Subsequent visual impairment during daytime, mainly loss of peripheral vision leading to tunnel vision, and loss of visual acuity, are progressive and result in legal blindness.[6]

While retinal dystrophy is usually classified as retinitis pigmentosa (RP), a subcategory of rod-cone dystrophy, other retinal dystrophies such as cone-rod dystrophy, choroidal dystrophy or global dystrophy have been described in BBS patients as well.[7] Beside retinal dystrophy as a major feature, other ocular involvement in the form of astigmatism, cataracts, colour blindness, macular oedema, macular degeneration and optic atrophy are reported as minor features.[5]

Obesity and metabolic syndrome: Beales et al. reported overweight (BMI < 25 kg/m^2) in 72%, obesity (BMI < 30 kg/m^2) in 52% and morbid obesity (BMI < 40 kg/m^2) in 16% of patients after puberty. While birth weight is usually normal, children gain weight rapidly within the first year of life, resulting in truncal obesity in adolescence.[5, 8] It is reported as one of the most debilitating factors of BBS, with difficulties to manage because of limited physical activity and poor understanding of dietary restrictions due to learning difficulties.[9]

The rate of metabolic syndrome in obese BBS patients is higher compared to matched control groups, including higher levels of fasting blood glucose, triglycerides and systolic blood pressure, resulting in a high rate of cardiovascular morbidity in BBS patients.[10]

Learning disabilities and development delay: Beales et al. classified learning difficulties as a primary feature, with 62% prevalence in their study cohort. Half of the patients attended a special school due to these difficulties and/or visual impairment. Development delay was classified as a secondary feature, with a delay of reaching motor milestones and speech development. Walking was delayed up to a year and speech development up to two years in about half of the patients. [5]

Various subsequent studies have measured intellectual disabilities using standardized neuropsychological tests. Kerr et al. reviewed adaptive functioning and verbal intellectual reasoning abilities of 24 BBS patients, of which 20–25% met the diagnostic criteria for intellectual disabilities.[11] Brinckman et al. used Wechsler scale on 42 patients and found a borderline IQ in the range of 70–79, with a mean score of more than one standard deviation below the mean. However, older patients could complete less subcategories due to visual impairement.[12] Barnett et al. measured Full Scale IQ in 16 BBS affected children, of which only 17.6% were in the range of average IQ (> 80), while the majority were in the range of mild mental retardation (50 < IQ < 69).[13]

Hypogonadism: Beales et al. found secondary sexual characteristics in all adult patients. In males, hypogenitalism was found in 89% of patients, with delayed puberty and maldescensus testis in 31% and 13%, respectively. In females, mean age of menarche was 13.8 years. Apart from irregular menstrual cycles, no abnormalities of the reproductive system were noted, and three patients gave birth to unaffected children.[5]

Hypogonadism in males has since been a frequently reported feature of BBS, with only two reports in literature of BBS-affected patients fathering children. In females, genital abnormalities are observed less frequently and range from hypoplasia of ovaries, fallopian tubes, uterus and vagina to complete vaginal atresia.[14]

Renal tract anomalies and diabetes insipidus: Renal tract abnormalities were not included in the cardinal features originally described by Bardet and Biedl, but were later suggested as a sixth cardinal feature.[15] In 1999, Beales at al. included them as a cardinal feature,when they found structural abnormalities in 26 out of 57 patients who had undergone radiological investigations. In their study however, no assessment of renal function has been conducted.[5]

As of today, reported renal tract abnormalities include renal cysts, fetal lobulation, scarring, dysplastic kidneys, unilateral agenesis, horseshoe kidney, ectopic kidney as well as vesico-ureteral reflux and hydronephrosis. Renal function of BBS patients ranges from normal over

impairment in urine concentration and nephrogenic diabetes insipidus to end-stage chronic kidney disease (CDK). Urinary tract infections are another frequently reported feature.[5, 15]

CDK is a major cause of morbidity and mortality in BBS patients. The onset of primary CDK is in early childhood with a rapid progression to end-stage renal disease (CDK 4–5). Patients who do not develop primary CDK retain near normal kidney function into adulthood, where a majority develops secondary CDK due to the risk factors (hypertension and diabetes) related to the disorder. Forsythe et al. reported the prevalence of CDK (2–5) with 31% and 42% in children and adults, respectively, and the prevalence of end-stage CDK (4–5) with 6% and 8%.[16]

1.1.3 Secondary features

In addition to these above-mentioned features, others have been reported with varying frequencies. These include neurological abnormalities (ataxia and spasticity, hearing loss), behavioral maladjustments and psychiatric disorders (autism-spectrum disorders, psychosis, depression), facial dysmorphisms (dental crowding, hypertelorism, retrognathia), cardiovascular anomalies (left ventricular hypertrophy, congenital heart disease), hepatic fibrosis, atresia ani and Hirschsprung disease. [1, 5, 14]

1.1.4 Diagnosis

BBS is usually not diagnosed before the start of vision loss, as the early symptoms polydactyly and obesity are unspecific. Mean age of diagnosis is nine years.[5]

If the diagnosis of BBS is made or highly suspected, patients should be assessed for baseline organ functions and screened for malformations. Initial assessments should include the eyes (visual function, electroretinogram, screening for refractive errors and strabism), kidneys (renal function, renal ultrasound, screening for diabetes insipidus) and metabolic function (glucose metabolism). Every patient should be once assessed for hearing loss, evaluated in development and educational status and reviewed by a clinical psychologist. If necessary, referrals to a cardiologist, orthodontist, speech therapist or mental health services should be made.

In addition, every patient should be once reviewed by a clinical geneticist to confirm the diagnosis by molecular analysis. Genetic counselling should be offered to affected families.

Annual assessment should include weight, blood pressure, function of liver, thyroid and kidney, glucose and lipid profile, an ophthalmology review (including an electroretinogram if the patient is under five years old) and an endocrinology review.[1]

1.1.5 Management

As there is no targeted therapy available, management of BBS is symptomatic and focuses on managing hypertension, diabetes and metabolic syndrome to limit the progress and prevent further damage to eyes and kidneys.

Aggressive weight control is an important part of disease management, but it often represents a continuous struggle for a majority of BBS patients. There is no causal medical treatment, as the pathomechanisms leading to obesity are still only partially understood. Some patients use antiobesity medication or elect to have bariatric surgery, such as gastric bypass, gastric banding or sleeve gastrectomy.[17] Dietetic input provides the safest weight loss strategy, but dietary restrictions are often hindered by learning difficulties and reduced physical activity.[9] Furthermore, the inability to manage obesity leads to a stigma placed on patients and parents by their environment as well as by attending physicians, which may additionally lead to bullying, depression and eating disorders. [18]

Renal-replacement therapy, such as transplantation or hemodialysis, is required in about 8% of patients due to the development of end-stage CDK. Although extra-renal comorbidities may complicate long-term patient management, renal transplantation has been shown to have a favourable outcome in young BBS patients.[19]

In the management of other aspects of BBS, treatment does not differ from the management of the same symptoms from non-syndromic causes. This includes an aggressive management of metabolic syndrome (including life-style modifications, oral antidiabetics, insulin, antihypertensive and antihyperlipidemic drugs) to reduce cardiovascular morbidity, hormone replacement therapy in cases of hypogonadism or other endocrine malfunctions, and corrective surgery for polydactyly and urinary, genital or cardiac malformations.[20, 21]

1.1.6 Epidemiology

The worldwide prevalence of BBS is hard to estimate, as it has been studied only in a few populations. Reported prevalence ranges from 1:650000 in Tunesia[22], 1:600000 in Switzerland[23], 1:125000 in the UK[24], over 1:18000 in Newfoundland[25], 1:13500 in Kuwait

Bedouin[26] to 1:3700 on the Faroer island [27]. The higher rates can be found in societies that have been historically isolated or where consanguineous marriages are common, or both.

Life expectancy is reduced in individuals with BBS but differs greatly due to the variability in organ involvement and the accessibility of appropriate health care. In a study conducted 1996 in Denmark, mean life expectancy was 46.4 years for women and 43 years for men, with a life expectancy of the general population of 63.8–77.4 years for women and 62–71.4 years for men.[28]

1.2 Genetics

1.2.1 Mode of inheritance

From initial pedigree observations, BBS was thought to be a monogenetic autosomal recessive disease. First mapping studies supported an autosomal recessive mode of inheritance but failed to map all families to the same locus, suggesting non-allelic heterogeneity. While 50% of families mapped to the same locus on 11q13, termed *BBS1*, five different loci were mapped before the first gene was identified.[29] This gene *MKKS* was known as the cause of McKusick-Kaufman syndrome (MKKS), an autosomal recessive syndrome sharing phenotypical features with BBS (postaxial polydactyly, congenital heart disease and hydrometrocolpos). Mutations in *MKKS* were independently identified to cause BBS in two different families, thus terming it *BBS6* as the first discovered BBS gene.[30,31]

Further genes were discovered in linkage studies of large multiplex families using positional cloning, and by combining homozygosity mapping in small families with bioinformatical comparison of protein structure, comparative genomics and protein expression studies.[32] Since 2010, further BBS genes have been identified in linkage studies of small families using whole exome sequencing.[33, 34]

To date, 24 genes have been identified, accounting for approximately 80% of all clinically diagnosed cases. An overview of all known BBS genes can be found in Table 2.

BBS#	HGNC name	Contribution to BBS morbidity in % [1, 16, 35, 36]	Sources
BBS1	*BBS1*	16.9–38.3	Beales et al. (2003)[37]
BBS2	*BBS2*	8–12	Nishimura et al. (2001)[38],
BBS3	*ARL6*	0–5	Chiang et al. (2004)[39]
BBS4	*BBS4*	0.6–2	Mykytyn et al. (2001)[40]
BBS5	*BBS5*	0.4–2.4	Li et al. (2004)[41]
BBS6	*MKKS*	1.7–6	Slavotinek et al. (2000)[30]
BBS7	*BBS7*	2–2.4	Badano et al. (2003)[42]
BBS8	*TTC8*	1–2.4	Ansely et al. (2003)[43]
BBS9	*BBS9*	1.2–6	Nishimura et al. (2005)[32]
BBS10	*BBS10*	14.9–21.7	Stoetzl et al. (2006)[44]
BBS11	*TRIM32*	-	Chiang et al. (2006)[45]
BBS12	*BBS12*	4.6–10.8	Stoetzel et al. (2006)[46]
BBS13	*MKS1*	-	Leitch et al. (2008)[47]
BBS14	*CEP290*	-	Leitch et al. (2008)[47]
BBS15	*WDPCP*	-	Kim et al. (2010)[48]
BBS16	*SDCCAG8*	-	Otto et al. (2010)[33]
BBS17	*LZTFL1*	-	Marion et al. (2012)[34]
BBS18	*BBIP1*	-	Scheidecker et al. (2014)[49]
BBS19	*IFT27*	-	Aldahmesh et al. (2014)[50]
BBS20	*IFT172*	-	Schaefer et al. (2016)[51], Forsythe et al. (2018)[52]
BBS21	*C9orf37*	-	Heon et al. (2016)[53]
BBS22	*NPHP1*	-	Lindstrand et al., (2014)[54]
BBS23	*IFT74*	-	Lindstrand et al. (2016)[55], Niederlova et al. (2019)[36]
BBS24	*SCAPER*	-	Niederlova et al., (2019)[36]

Table 2: List of BBS genes. HGNC (HUGA Gene Nomenclature Committee) name, contribution to BBS morbidity and identification paper.

1.2.2 Genetic heterogeneity

BBS1 and *BBS10* are considered the major contributors, together accounting for about 45% of cases. Other genes such as *BBS15/WDPCP*, *BBS18/BBIP1* and *BBS19/IFT27*, were found mutated only in a single family or even a single patient. [48, 49, 50] There are, however, regional differences in the genetic spectrum, and some genes are found mutated more frequently in certain populations than in others.

BBS1 and *BBS10* are reported to contribute about 23% and 21%, respectively, to the mutational spectrum of patients with European descent.[37, 44] This frequency is lower within patients from Saudi Arabia, where the major contributors are *BBS1*, *BBS4* and *BBS3*.[56] In Indian patients the major contributor is *BBS3*, followed by *BBS10*, *BBS9* and *BBS2*.[57]

However, the genetic heterogeneity of BBS exceeds 24 involved genes, as over a few hundred different mutations have been found, demonstrating a high allelic heterogeneity and encompassing missense, nonsense, small indels, splice site mutations and large deletions. The percentage of mutations that are private, reported only in a single family, ranges from 62–100%.[58]

Apart from private mutations, there are few that are recurrent in various cohorts: One *BBS1* missense mutation, Met390Arg, constitutes around 80% of *BBS1* mutations, and significantly increases the contribution of *BBS1* alleles to the general mutational load. This allele is almost exclusively seen in families with European origin.[37, 58] Another recurrent mutation C91fsX95, a single base insertion in *BBS10* leading to premature protein termination, made up 46–54% of mutant alleles, likewise increasing the contribution of *BBS10* to the mutational load. This mutation is predominantly detected in families with European origin, but has also been reported in families of Afghan and Turkish origin.[44, 58] Haplotype analysis indicated a common founder in both recurrent alleles.[37, 58]

1.2.3 Founder effect

Founder effect and genetic drift were thought accountable for the high prevalence of BBS in some isolated populations, whose population descended from few common ancestors some centuries ago. On the Faroe Islands, with the highest prevalence worldwide (1:3700, 13 patients in a population of 48000), this assumption has been proven to be correct, when a single splice site mutation in *BBS1* (c.1091+3G>C) was found among all patients. All but one patient were homozygous for the mutation, while the remaining patient was compound heterozygous. This patient also carried the recurrent Met390Arg mutation and had one parent not native to the islands. The allele frequency of this founder mutation was 1:38 in the general population of the islands.[27]

Another founder mutation has been found in the North American Hutterites, a population of about 40000, descending from fewer than 100 common founders in the 16th century. In four patients from two distinct, genetically isolated groups of Hutterites, the same novel splice site mutation in *BBS2* (c.472-2A>G) was identified, with a common haplotype of 1–6 Mb.[59]

Similar preconditions can be found in the Newfoundland population, in which many Mendelian disorders have been studied. In 1999, a large Newfoundland pedigree was used to map the locus of *BBS5*.[60] However, sequencing of the first discovered BBS genes revealed nine different mutation and at least six different genes in Newfoundland BBS patients,

indicating that the unusually high prevalence of 1:18000 cannot be attributed to founder effect alone.[25]

1.2.4 Triallelic inheritance

While BBS was considered an autosomal-recessive disease, Katsanis et al. proposed a different mode of inheritance: In 2001, after the identification of the first two genes (*BBS6* and *BBS2*), they suggested that BBS is inherited in a triallelic-digenic mode, in which three mutations in two different genes are necessary for disease manifestation. This proposition was based on a cohort of 163 patients, in which both genes were analyzed for mutations. In three families, three mutations in these two genes were found, which segregated with disease in the family. In one of these families, an unaffected sibling harbored the homozygous change in *BBS2*, but was wild type for *BBS6*, while the affected sibling harbored both changes in *BBS2* and one in *BBS6*.[61]

Similar results were reported after the identification of *BBS4*.[62]

Beales et al. supported the theory of triallelic inheritance by studying *BBS1* mutations in a large cohort. They found multiple families in which three alleles segregated with the disorder. Furthermore, two families were identified in which the recurrent Met390Arg allele was found homozygous in unaffected individuals. This non-penetrance of a bona-fide mutation might be due to the presence of a third protecting allele, or the absence of a third required allele for pathogenesis.[37]

Badano et al. proposed an adapted model of triallelic inheritance, suggesting that the third allele was not necessary for disease manifestation, but acts as a modifier and might exacerbate the phenotype. They found a significant increase of severity of the phenotype in affected individuals with three pathogenic alleles compared to affected sibling with only two, who differed in severity and age of onset of various aspects of the phenotype. This modifying effect of non-segregating BBS-allele on the phenotype may explain the high intra- and interfamilial variability of BBS.[63]

1.3 Pathophysiology

The underlaying cause of the varying symptoms seen in BBS is a defect of the primary cilium. This cell organelle is a single organelle protruding from the surface of almost all cell types. It was first described in 1867 by Alexander Kowalevsky, but long believed to be vestigial and with no remaining function. Today, the primary cilium is known as an

important sensory cell organelle that responds to mechanical and chemical stimuli and communicates these stimuli to the cells interior.[64] As such, is plays a crucial role in cell signaling during development and homeostasis especially in embryonic patterning and organ development. Defects in cilia assembly or function result in the heterogenous group of disorders known today as ciliopathies.[65]

1.3.1 The primary cilium

Like the motile, secondary cilium, the primary cilium is a protuberance of the cell membrane supported by the axoneme, a microtubule-based cytoskeleton. The microtubules of the axoneme are arranged circularly in nine doublets. While the axoneme of the motile cilium has two microtubules in the center and is equipped with the motor protein dynein, the primary cilium is lacking these structures and the ability to move. Like their motile counterpart, the primary cilium is rooted in a basal body, which is derived from the mother centriole of the centrosome, while the daughter centriole is located in a 90 degree angle to the other and surrounded by electrodense material, the pericentriolar matrix.[66]

The basal body is shaped like a hollow cylinder formed by nine microtubule triplets, of which the innermost and middle form the anchor from which the doublets of the axoneme originate. The area where the basal body microtubules converge to the axoneme tubules is known as the transition zone. Here, the basal body is connected to the cell membrane by transition fibres, which form a "ciliary gate" separating the cellular from the ciliary lumen and controlling the entry and exit of proteins and lipids to the cilium. While the ciliary membrane is continuous with the cell membrane, its composition is distinct, with a variety of transmembrane receptors to receive external stimuli and molecules within the cilium to transduce these signals along signaling cascades into the cells interior.[65] (Figure 3, page 23)

Primary cilia are only present on non-cycling differentiated cells or stem cells in G_0 phase. During cell division, the centrosome is resorbed, duplicated and facilitates the organization of the mitotic spindle. After cell division is completed, the centrosome detaches and the mother centriole migrates to the apical surface of the cell, initiating the assembly of a single primary cilium.[67]

1.3.2 Intraflagellar transport

As protein translation does not occur in the ciliary lumen, proteins required for assembly and function have to be transported there. This is facilitated by intraflagellar transport (IFT), a highly conserved process of motility that can be found in almost all eukaryotic cilia and

flagella. In this process, protein complexes known as IFT particles, consisting of multiple IFT proteins, dock at the transition zone, load their cargo and are transported along the microtubule by motor proteins. Anterograde transport to the ciliary tip is powered by the motor protein kinesin-2 while retrograde transport to the ciliary base is powered by dynein.[65]

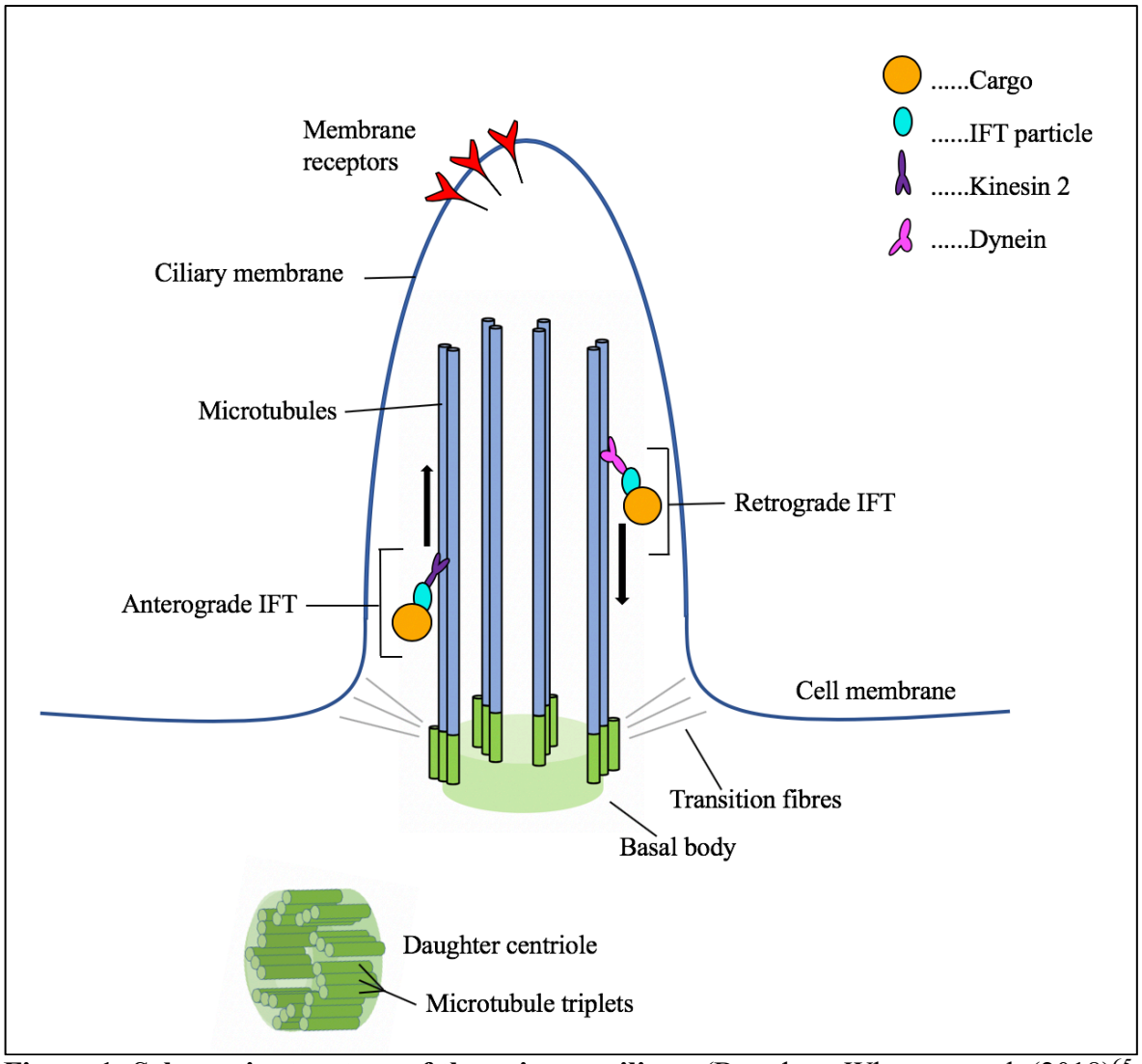

Figure 1: Schematic structure of the primary cilium. (Based on Wheway et al. (2018)[65] and Lüllmann-Rauch, R. (2012) Taschenlehrbuch Histologie[66]. (Illustration created in PowerPoint).

While structural proteins like tubulin are bound directly to IFT particles for transport, most membrane proteins do not. They need to be recognized by a trafficking signal, packed into membrane coats and loaded onto IFT particles for transport. This process is facilitated by a protein complex formed by seven proteins coded by BBS genes. BBS1, BBS2, BBS4, BBS5, BBS7, BBS8 and BBS9 form a complex named the BBSome.[68] Another protein complex, consisting of the BBS proteins that show structural homology to chaperone proteins (BBS6,

BBS10 and BBS12), is required for the assembly or stability of the BBSome and is called the BBS chaperone complex.[69]

Another protein that is required for the function of the BBSome is BBS3**,** also known as ARL6, a small GTPase that effects the binding of the BBSome to liposomes and is required for the formation of a distinct polymerized coat. The BBSome-ARl6 complex has been located to vesicular patches along the microtubule axoneme. Without ARL6 function, the BBSome can be correctly assembled but is not able to localize to the cilium.[70]

While the molecular mechanism of how the BBSome works is still unclear, certain proteins have been identified as its cargo. These are G-protein coupled receptors like neuronal receptors Somatostatin-Receptor 3, Dopamine-Receptor 1, Melatonin-concentrating-hormone receptor 1[68] and transmembrane receptors working in sonic hedgehog signalling, Smoothened (Smo) and G-protein coupled receptor 161. The Leptin receptor was also proposed as possible cargo.[36]

The genes found mostly mutated in BBS patients are either coding for the BBSome or the chaperone complex, or *BBS3*.[36] Mutation in other genes contribute a small percentage to BBS morbidity and some have been primarily associated with other ciliopathies than BBS. *BBS13,* also known as *MKS1,* is the cause for the lethal multi-systemic ciliopathy Meckel-Gruber syndrome (MKS, MIM: 249000), as well as Leber congenital amaurosis (LCA, MIM: 611755), a hereditary retinal dystrophy.[65] *NPHP1/BBS22* is a common cause of isolated Nephronophtisis (NPHP, MIM: 256100) and also found in Joubert syndrome (JBTS, MIM: 609583).[55] Other genes code for centrosomal proteins (*SDCCAG/BBS16*[33] and *BBS14/CEP290*[71]), BBSome interacting proteins (*LZTFL1/BBS17*[34] and *BBIP1/BBS18*[49]), IFT proteins (*IFT172/BBS20*[72]) or proteins of yet unknown function (*C8orf37/BBS21*[53]*).*

Why defects in BBSome and the associated proteins lead to the exact pathologies observed in Bardet-Biedl Syndrome is only partially understood. Molecular mechanisms potentially causing obesity, polydactyly and retinitis pigmentosa have been identified, while the pathophysiology of renal and reproductive system anomalies and other minor features of the syndrome is yet to be discovered.[36]

1.3.3 Pathomechanism of polydactyly

It is assumed that polydactyly and other limb defects result from a defect in the Hedgehog signaling during development.[36] This pathway is the best understood signaling pathway

during embryogenesis and takes place in the primary cilium. While it is quiescent in most adult tissues, it regulates body patterning and organogenesis of the nervous system, the lung, kidneys and various other organs. Misfunction of the pathway leads to left-right patterning and limb defects as well as neural tube defects.[73]

While components of the signalling pathway have been identified as cargo of the BBSome, it is not understood how malfunctions in ciliary trafficking lead to limb defects. As polydactyly is not reproduced in BBS mouse models, Marwan et al. studied the fin bud development of BBS mutant zebrafish. As the pectoral fin and the axial limb of mammals is derived from a common homologue, changes seen in the development of the fin might reflect limb defects in BBS patients. Marwan et al. detected an increase of Sonic Hedgehog expression in the developing fin bud and subsequently changes in the skeletal elements of the fin.[74]

1.3.4 Pathomechanism of retinal dystrophy

In the retina, primary cilia are an essential organelle of photoreceptors. Both cone and rod cells are made up of an inner segment close to the perikaryon, where metabolism takes place, and an outer segment containing Rhodopsin-membrane discs, where the phototransduction takes place. Both segments are connected by a modified primary cilium, the connecting cilium. Its structure resembles the primary cilium, with the basal body located at the apical side of the inner segment and the exoneme extending to the distal end of the outer segment. While light reception is initiated by the stimulation of Rhodopsin in the outer segment, the transduction cascade to the inner segment and the body of the cell is facilitated by two other proteins, which are transported retro- and anterogradely through the connecting cilium by IFT.[75, 76]

While cilia assembly is not as reliant on BBS-related proteins as cilia function, BBS mutant mouse models showed normal retina structure and photoreceptor development at birth. Progressive loss of the outer segment occurred, leading to apoptosis and photoreceptor cell death before the age of 10 months. In the retina of these mutant mice, vesicles were observed to accumulate at the base of the cilium and rhodopsin accumulated in the inner segment instead of correctly localizing to the outer segment. These changes seemed to lead to apoptosis with progressive loss of photoreceptors and vision, similarly as it can be observed in BBS patients.[75]

1.3.5 Pathomechanisms of obesity

Obesity in BBS appears to have a dual origin. Both a dysregulation of the central nervous mechanisms in energy homeostasis as well as changes in adipogenesis have been observed in BBS and attributed to a misfunction of primary cilia.[36, 1]

In the central nervous system, the hypothalamus is the key neuronal structure to control long-term energy homeostasis and body weight. It consists of several nuclei, of which the arcuate nucleus (ARC) has been intensely studied. The neurons of the ARC are heterogenous and can be divided in groups based on their expression, namely POMC neurons (expressing proopiomelanocortin) and NPY neurons (expressing neuropeptide-Y). These groups work antagonistically, as the stimulation of POMC neurons increases appetite (orexigenic effect) and the stimulation of NPY neurons decreases appetite (anorexigenic effect).[77] The ARC has a leaky blood-brain barriers and is likely to be exposed to circulating nutrients, short-term satiety hormones and adiposity hormones.[78] Misregulation of both short-term satiety signalling and long-term adiposity feedback signalling has been associated with the primary cilium and with obesity in BBS.

Short-term satiety hormones are polypeptides secreted from gastrointestinal endocrine cells after food intake. About 20 are known that act synergistically in various areas of the brain to suppress appetite and food intake. [78] Among them is peptide tyrosine tyrosine (PYY), that activates neuropeptide-Y receptors, influences gastric mobility and intestinal adsorption and reduces appetite.[79] Loktev et. al. identified the neuropeptide Y receptor type 2 in ARC neurons to be cilium-dependent and its signalling to be defective in BBS mutant mice. This receptor inhibits signalling in ARC neurons and leads to a decrease in orexigenic NPY and an increase in anorexigenic POMC expression. In BBS18 deficient mice, the ARC neurons primary cilia lacked the neuropeptide Y receptor type 2 and failed to respond to the anorexigenic function of its ligand PYY3-36. In these mice, an increase of food intake, body weight and obesity was observed.[77]

In contrast to short-term satiety signals which are secreted during meals, adiposity signals provide an ongoing signal to the brain. The main adiposity signal is leptin, which is produced by adipose tissue and released into the bloodstream proportional to fat mass. In the hypothalamus, it promotes weight loss by decreasing food intake and increasing energy expenditure. BBS patients show a high level of serum leptin as well as central leptin resistance. In comparison with healthy control subject who have the same BMI, BBS patients

have more fat mass, especially visceral fat, and higher levels of leptin. In BBS mice model in which obesity is reproduced, the molecular mechanism of leptin resistance differs from that of diet-induced resistance, and eliminating obesity and hyperleptinemia does not restore leptin sensitivity in BBS mice. This suggests that defective leptin signaling is not secondary to obesity in BBS but contributes to its development.[80]

In addition to central nervous mechanisms of weight control, primary cilia have also been associated with adipogenesis. Adipocytes differentiate from mesenchymal stem cells, which commit to the adipocyte lineage by differentiating into preadipocytes before their terminal differentiation into adult adipocytes. Both preadipocytes and adult adipocytes are non-ciliated, but the differentiating preadipocytes express a transient cilium. One major signaling pathway to inhibit adipogenesis is the Wingless (Wnt) pathway, whose receptor is located in the cilium. Signaling of this pathway maintains preadipocytes in an undifferentiated state, and upon suppression adipogenesis is initiated. In BBS knockout of human preadipocytes, the number of ciliated cells decrease, and the proadipogenic signaling pathways are increasingly activated. In addition, fat accumulating cells derived from dermal fibroblasts from two BBS patients showed a significant increase of triglyceride levels in their fat vacuoles and higher secretion of leptin, compared to cells derived from healthy controls.[81]

1.4 Consanguinity and autosomal recessive disease

1.4.1 Consanguinity

Consanguinity is defined as a union between persons who are related, usually second cousin or closer. About 20% of people worldwide live in communities that favour consanguineous marriage, and about 8,5% of babies are born to consanguineous parents.[82] While in most western countries, consanguineous marriages were socially accepted and favoured especially by the ruling class, the general attitude towards it shifted in the mid-18th century.[83] While such unions are customary in many countries in the Middle East and South Asia (picture), the highest rates can be found in Kuwait (68%), Saudi Arabia (56%) and Pakistan (55-59%).[84] Advantages of consanguineous marriages are more stable families (strengthening of family ties with the extended family, greater marriage stability, less marriage disharmony), better status and greater autonomy of the woman, the economic advantage of a reduced dowry and no dispersal of wealth and landholdings, as well as network to care for old and disabled members of the family.[82, 83,

If two people share a common ancestor, their share a fraction of their autosomal DNA. A closer relation results in a bigger identical fraction. Statistically, second cousins share 1/32, first cousins 1/8 and double first cousins 1/4 of their DNA. If a child inherits the same segment from both parents, that segment will be homozygous, as it is identical by descend (IBD). The likelihood that their child is homozygous for any given region of their DNA is 1/64 for second cousin, 1/16 for first cousins and 1/8 for double first cousins. This likelihood is expressed by the coefficient on inbreeding F. To calculate F, the closest relation between parents is used, under the assumption that all grandparents are only distantly related. In cultures where consanguineous marriages are common, the closest relationship between parents is often not the only one as they are multiple times related through various generations. This increases the degree of homozygosity in their offspring.[85]

1.4.2 Autosomal recessive disease

Most people carry one or more variants for an autosomal recessive disease.[86] The probability to find a partner carrying a variant for the same disease is very low in a population where partners are chosen randomly and is determined by the allele frequency of the recessive variant. In an offspring of parents sharing a common ancestor, the probability to inherit the same variant from both parents increases. The closer related the parents are, the more DNA is identical between them, and the probability to pass on the same variant increases. As first cousins statistically share 1/8 of their DNA, their offspring is homozygous for 1/16 or 6.25%. Thus, the risk to be homozygous for an autosomal recessive disease is statistically 6.25 times higher if ones parents are first cousins than if they are not related.[87]

Generally, the prevalence of serious birth defects and congenital disease is 2–4%, and in offspring of first-cousins it is roughly doubled.[87] Various studies have tried to measure the empirical increase of autosomal recessive disease and other congenital malformations in children of consanguineous parents.[82]

In a long-term study conducted in Birmingham which followed children for 5 years after birth, babies of all ethnic groups except Pakistani had a 3.89% rate of serious malformations leading to death or chronic disability, while for Pakistani babies it was 10.2%. Most of the excess resulted from an increase of autosomal recessive disease, which contributed 0.4% of chronic diseases in all ethnic groups combined except Pakistani, and 6.3% of chronic disease in Pakistani children. Other than recessive disease, there was also an increase in cardiac malformations. [88]

The Born in Bradford study with a follow-up of one year studied different risk factors such as deprivation, alcohol consumption, smoking, maternal age and education as well as parents' relation. It determined consanguinity in parents to be the most significant risk factor. They observed a frequency of congenital and genetic malformation of 2.3% in all births of unrelated White British couples, 2.6% in unrelated Pakistani couples, but 6.2% in Pakistani first cousin couples.[89, 90]

1.4.3 Genetic counselling

Social customs should be taken into account when counselling families at risk of recessive disorders. Discouraging consanguineous marriage on the ground of an increased risk in their offspring has proven to be unsuccessful, with negative reactions in the affected community and no effect on marriage choices. To make informed partner choices, it is necessary for affected families to understand the relationship between recessive disease and consanguinity.

Darr et al. have identified the extended family to be the network in which individuals at risk could be identified and which can be used to pass on information. When an affected child is diagnosed in a family practicing consanguineous marriage, or an affected relative identified by taking an extended family history, the nuclear family should be counselled by adequately trained professionals. Only when adequate information is comprehended by the affected family, they can pass it on to the extended family, who can make use of genetic counselling and carrier testing. In summary, to enable affected families to make informed choices, genetic counselling must work within these social structures rather than oppose them.[82]

1.4.4 Disease gene identification and diagnosis

To identify the gene for an autosomal recessive disease in an individual from consanguineous parents, homozygosity mapping is utilized. It is based on the assumption that a homozygous mutation in a recessive gene is inherited from an ancestor to the individual twice, once through the maternal and once through the paternal line. The mutation is therefore identical by descent, as well as a chromosomal segment surrounding the mutation. Variable DNA sequences within this segment, such as short tandem repeats (STR) or single nucleotide polymorphisms (SNPs), will be homozygous as well. The less generations part the ancestor from the affected individual, the less of the surrounding segments have been recombined by crossing over. Thus, the homozygous segment will be bigger if the relationship between the parents of the individual is close.[91, 92]

To look for the cause of autosomal recessive disease in a chromosomal segment that is identical by descent, so called runs of homozygosity (ROH) can be mapped by analyzing variable markers distributed over the whole genome. From these regions, candidate genes can be selected for sequencing.[92] On the other hand, if the genes for a certain disorder are known, analyzing markers in the proximity of these genes can indicate whether identity by descent is likely or can be excluded.[35] Two groups of markers are widely used for these analysis, namely microsatellite markers and SNPs.[92]

Microsatellite markers

Microsatellites, one type of short tandem repeats (STR), are repetitive DNA sequences that are distributed over the genome. They consist of a short motive of nucleotides, mostly a dinucleotide, that is repeated in tandem several times. Due to a relatively high mutation frequency, different alleles of the same marker can be observed in the population, a phenomenon which is called polymorphism. This mutation occurs because the repetitive elements cause the polymerase to slip, thus adding repeats to the sequence.[93] The result is that in the population there are many alleles for one locus, whose allele size varies in discrete steps. As up to 10 alleles are known for single locus, they are more informative than SNPs, who exhibit only two different alleles, seldom three.[94]

To establish the allele size of microsatellites, primers flanking the variable regions of the repeat sequence are designed. The region is amplified by PCR and amplicon size is analyzed by electrophoresis. Alternatively, primers can be fluorescently labelled, and amplicons size can be analyzed by capillary electrophoresis of conventional sequencers. Multiple loci who differ in allele size range or labeled with a different fluorescent dye can be amplified in the same reaction.[93] Panels consisting of 300–400 markers were used as standard procedure for genotyping. However, their resolution is too low to study runs of homozygosity in non-consanguineous families. Thus, they have gradually been superseded by SPNs analysis.[82]

Single nucleotide polymorphisms

Variation in single base pairs (bp) make up most of the 0.1% of the genome that differs between individuals. They are distributed randomly over the genome, with a rate of one SNP every 300–1000 bp.[95]

Today, hundred thousand of SNPs are genotyped parallelly on chips, so called SNP arrays. For each SNP that is detected with the array, two oligonucleotide probes are fixed on a chip,

with the 3' end ending next to the SNP. Amplified DNA and fluorescently labeled didesoxinucleotides (ddNTPs) are added. A DNA fragment binds to each oligonucleotide and one ddNTP is added at the 3' end, complementary to the SNP allele of the DNA. Fluorescence is then detected by laser. With both alleles for each SNP represented on the chip, the genotype of the SNP can be detected as homozygous either for the major or minor allele, or heterozygous.[96]

Next generation sequencing

With the introduction of next generation sequencing (NGS), or massively parallel sequencing, homozygosity mapping and sequencing can be performed in one step. The introduction of NGS means a major reduction in time and cost of sequencing, but a massive increase in sequencing throughput creates difficulties in data management and variant filtering. As a result, targeted sequencing of all the coding regions has been established as standard procedure for most applications. Whole exome sequencing (WES), which covers about 1–2% of the genome, yields about 20000 variants per individual, while whole genome sequencing (WGS) yields as much as 4 million variants.[97, 98] As 85% of mutations related to Mendelian disease are located in exon or splice sites, WES substantially reduces time and cost of sequencing as well as data management while keeping loss of information comparably low.[99]

Various sequencing platforms are in use today, each updated frequently to keep up with changing demand and technical possibilities. They all require short template DNA that is sequenced parallelly during synthesis. While most platforms rely on amplification before sequencing, some platforms sequence individual DNA molecules directly. Generally, the process can be divided into three steps:

1) In the first step, a library of DNA templates is prepared: Genomic DNA is fragmented, the fragment ends are modified by adding adapter sequences and finally amplified. If only a subset of the genome is sequenced, regions of interest are captured by hybridizing to complementary probes.
2) In the second step, individual DNA fragments are spatially separated, for example on a flow cell, and amplified to form a cluster of identical DNA templates. Sequencing is performed on each DNA template simultaneously by detecting the nucleotide that is incorporated during synthesis.[100]

3) As the separate reads generated for each cluster are a few hundred to one thousand base pairs in lengths, they have to be aligned against the reference genome to create a continuous sequence. Deviations from the reference sequence, such as SNPs, short deletions and insertions, and large deletions like copy number variants, are identified, and variants are annotated by adding information from databases to include gene name, amino acid change and other information. The final list of annotated variants is filtered and presented in a spreadsheet.[99,101]

To determine runs of homozygosity from NGS data, variant data can be uploaded into the web based HomozygozityMapper.[102]

2 Material and Methods

2.1 Families

Two consanguineous families affected by Bardet-Biedl syndrome were recruited by Muzammil Ahmad Khan from the Rehmani-Khail village in the rural area of Dera Ismail Khan district, in the Khyber Pakhtunkhwa Province of Pakistan. Prior to recruitment, approval was obtained from the ethical review board of the Gomal University in Dera Ismail Khan city (Institutional Review Board number 04/ERB/GU). Both families were of Pashtoon origin but apparently not related. The participants or their guardians were informed about the study scheme and written consent was obtained. Pedigrees were drawn to establish the degree of consanguinity. In affected individuals, BBS associated symptoms were documented using a self-designed questionnaire, and peripheral blood samples were drawn for biochemical analysis (lipid profile, liver function tests and renal function test).[103] The DNA of affected and unaffected family members was sent to Graz.

2.1.1 Family BD9

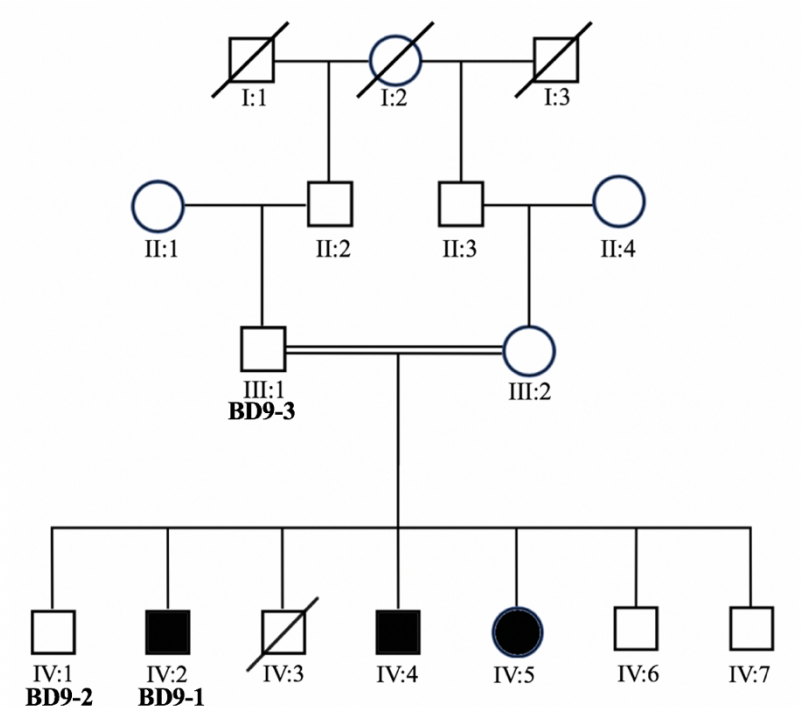

Figure 2: Pedigree of family BD9. Circles represent females, squares represent males. Black symbols indicate affected, white symbols indicate non-affected family members. Double lines between spouses indicate consanguinity. DNA was obtained from all "BD9-" indicated family members. (Pedigree created in PowerPoint)

In this family three children from a couple who were half cousins were affected by BBS. Four children were unaffected, of which one was deceased. Inheritance in this family agrees with an autosomal recessive mode of inheritance. DNA was obtained from one affected child, one unaffected child and their father. As the parents of the affected children shared one grandparent, they statistically share 1/16 of their DNA, resulting in an inbreeding coefficient (the probability that two alleles at any locus are identical by descent) of 1/32 in their offspring.

2.1.2 Family OPH22

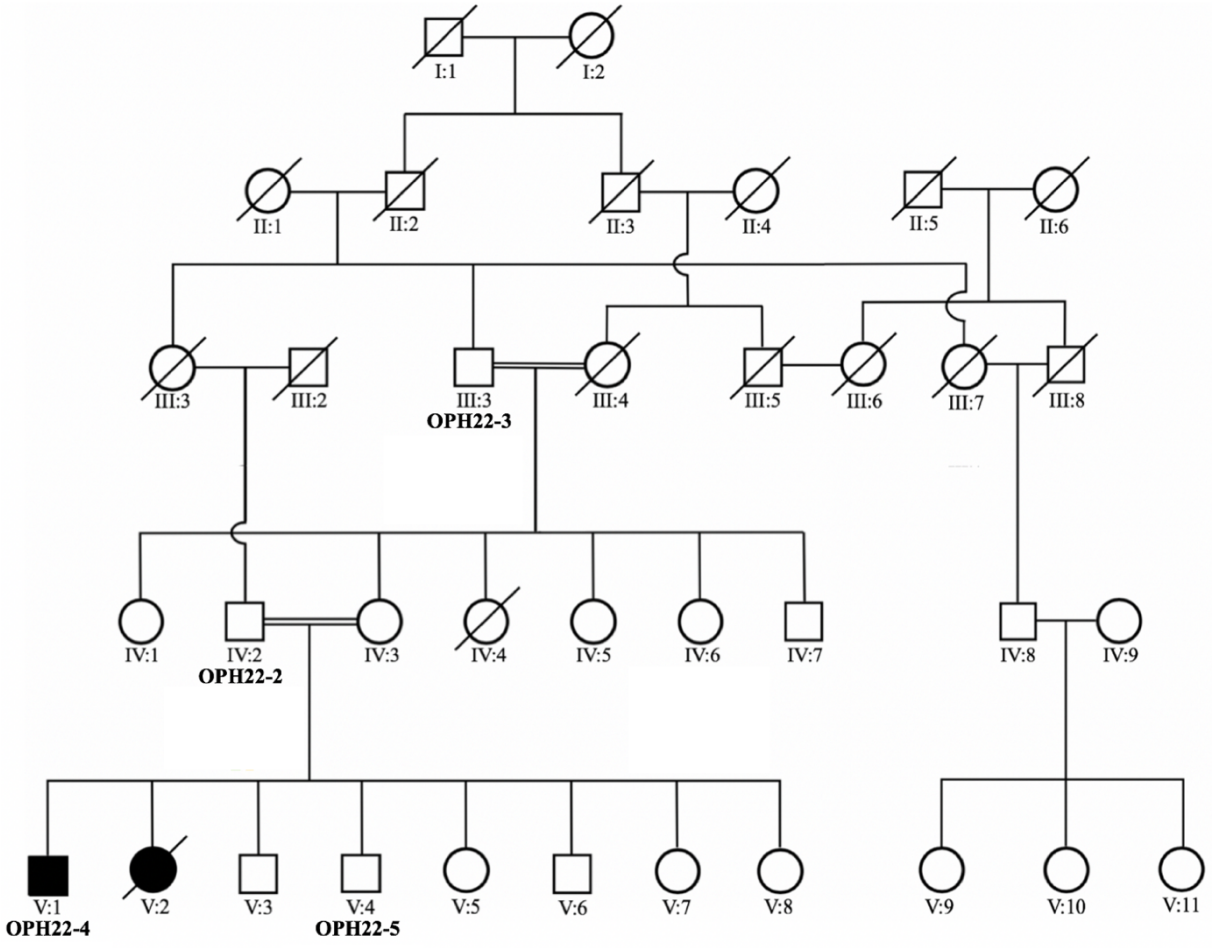

Figure 3: Pedigree of family OPH22. Circles represent females, squares represent males. Black symbols indicate affected, white symbols indicate non-affected family members. Double lines between spouses indicate consanguinity. DNA was obtained from all "OPH22-" indicated family members. (Pedigree created in PowerPoint)

In this family, two children of a first cousin couple were affected, of which one was deceased. Six children were unaffected. Inheritance in this family agrees with an autosomal

recessive mode of inheritance. The DNA was obtained from one affected child, one unaffected child, their father and their maternal grandfather.

As first cousins, the parents would share 1/8 (12.5%) of their DNA, resulting in an inbreeding coefficient of 1/32 (3.125%) in their children. However, as the mother was herself the offspring of a first-cousin union, the parents share 5/32 (15.625%) of their DNA and the coefficient of inbreeding in their children increases to 5/64 (7.8125%).

2.2 Microsatellite marker analysis of *BBS1* and *BBS10*

As *BBS1* and *BBS10* contribute about 45% of the mutational load of BBS, indirect genetic diagnosis by microsatellite marker analysis was carried out to establish whether identity by descent was likely or not. Two markers flanking *BBS1* and one marker in the close vicinity of *BBS10* were selected from the ABI Prism Linkage Mapping Set Version 2.5 MD10 (Applied Biosystems, US). Location of markers in relation to *BBS1* and *BBS10* are shown in figure 4.

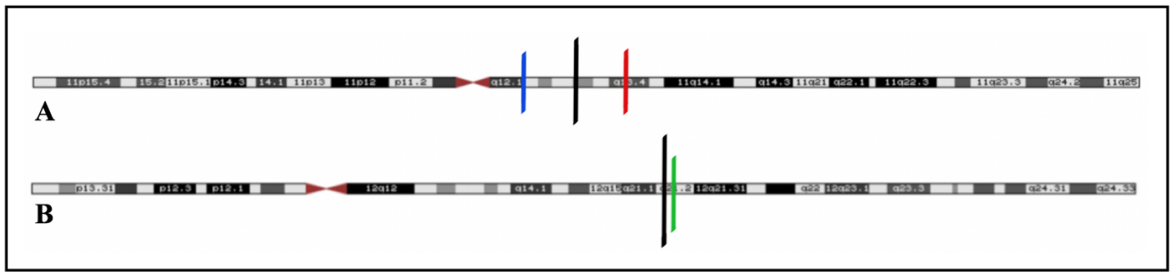

Figure 4: Relation of *BBS1* and *BBS10* to analyzed markers. A. Chromosome 11 with loci of *BBS1* (**black**) and markers D11S4191 (**blue**) and DS11S1314 (**red**). **B.** Chromosome 12 with loci of *BBS10* (**black**) and marker D12S326 (**green**) on chromosome 12. (Ideograms and loci were obtained from USCS genome browser [http://genome.ucsc.edu][104] and annotated in PowerPoint)

PCR mix was set up with genomic DNA of all individuals, Primers from ABI Prism Linkage Mapping Set and Hotstar Taq Mastermix (Quiagen, Netherlands), containing Taq DNA-Polymerase, PCR Puffer and dNTPs.

PCR mix contained:

- 0.5 µl genomic DNA
- 0.5 µl Primer (forward and reverse)
- 5 µl Hotstar Taq Mastermix
- 4 µl H_20

Reaction volume: 10 µl

Amplification was conducted using following cycling conditions:

Temperature in °C	Time	Note
95	15 min	
94 57 72	30 sec 90 sec 90 sec	28 cycles
72	20 min	

As allele size range of markers D11S1314 and D11S326 differ, PCR products for both markers were combined for each individual. 0.5 µl Gene Scan Liz Dye Size Standard was added. PCR products were then denatured in the thermocycler at 95°C for six min and stored on ice. Capillary electrophoresis was performed with ABI Prism 3130xl Sequencer and results analyzed with Peak Scanner^{TM} Software v. 1.0 (all from Applied Biosystems, US).

2.3 Whole exome sequencing

One affected individual (OPH 22-4, BD 9-1) from each family was selected for WES. Library preparations was performed with Nextera Rapid Capture Enrichment for the coding exome and sequencing was performed on Next Seq 550 (both from Illumina, US).

2.3.1 Library preparation

As Nextera Rapid Capture enrichment protocol uses enzymatic tagmentation of DNA, exact amount of DNA is needed. Genomic DNA was therefore quantified using Qubit 2.0 Flourometer and Quibit TM dsDNA HS Assay Kit (both from Invitrogen, US), according to manufacturer's instructions. Genomic DNA was normalized to 10 ng/µl, quantified again and diluted to 5 ng/µl. Total input DNA was 50 ng in a volume of 10 µl per sample.

Then, tagmentation of DNA was carried out. This process combines fragmentation of DNA and adapter ligation to the fragments ends and is mediated by the Nextera transposome, a modified derivate of a transposase.[105] Subsequently, tagmented DNA fragments were cleaned from the enzymes by hybridization to biotin magnetic beads and separated on a magnetic stand. DNA was then eluted from the magnetic beads. Sequencing adapters for subsequent amplification and indexing adapters to facilitate pooling of samples were added in 10 cycles of PCR. Separation of enzymes and samples was carried out as before.

Size analysis of DNA fragments was performed using Bioanalyzer Agilent DNA 7500 Assay (Invitrogen, US) according to manufacturer's instructions. Fragment size was between 150 and 1000 bp, peaking at approximately 300 bp, as required. Samples were then pooled.

Enrichment of coding exons was performed by hybridizing DNA to oligos targeting the coding exons in 10 cycles of PCR. Hybridized DNA was captured by adding streptavidin-coated magnetic beads, while non-hybridized DNA was removed by two heated washes on a magnetic stand.

For maximum specifity of the enrichment process, hybridization and capture were repeated. The enriched samples were then amplified by PCR and cleaned up as before. Fragment size was analyzed using Bioanalyzer Agilent DNA 7500 Assay, and library concentration was measured using Quibit TM dsDNA HS Assay Kit, as before. Fragment size ranged from 150 bp to 1000 bp, with a concentration of 91.4 mmol/l.

2.3.2 Sequencing

For the acquired concentration for sequencing, the DNA library was diluted with H_2O at to 4 ng/µl. Then, it was denatured with 0.2 N NaOH and diluted with 200mM Tris-HCl to a final concentration of 1.1 pM. 650 µl of the library were then pooled with two other libraries, loaded onto the reagent cartridge and sequenced on Next Seq 550.

2.3.3 Data analysis

Raw sequence data was aligned to the reference genome (GRCh37/hg19) using the BaseSpace™ application to generate BAM and VCF filed. Variant filtering was performed using VariantStudio Software (both from Illumina, US).[103]

Variants were filtered manually from excel spreadsheet using the following criteria:

- Selection of all variants located in known BBS genes
- Rejection of all variants not located in coding regions
- Rejection of all synonymous variants
- Rejection of all variants with a frequency over 1%
- Rejection of all variants that were predicted to be non-pathogenic by PolyPhen-2 and SIFT
- Rejection of all variants in heterozygous state

Parallelly, raw data was uploaded to HomozygocityMapper.[106] Runs of homozygosity were compared to loci of known BBS genes.

2.4 Sanger sequencing

Validation and segregation analysis of the candidate variant was performed by Sanger Sequencing. Sequencing was performed twice, first with primers that were on stock from a previous study, second with newly designed primers, which were designed with Primer3[107] and ordered from Microsynth AG (Switzerland).

Primer	Sequence
BBS9E4forward	5'TAA TGA ATT GTT TTG TTT ACT CAC AGT G3'
BBS9E4reverse	5' CCA AGT CAG CAC TAG GGA ATA TC 3'
BBS9_SB_f	5' TCA CAG TGG CTG TGG TTA TGA 3'
BBS9_SB_r	5' CCT GAG ACA GAG TAG ACA CAA AGT T3'

Concentration of DNA was determined from 1µl of each sample using NanoDrop 1000 (Thermo Fisher Scientific, US) spectrophotometer.

Individual	DNA in ng/µl
9-1	7.9
9-2	24.6
9-3	11.4
22-1	68.5
22-2	43.7
22-3	30.3
22-4	25.9

PCR was set up:

- 2 µl DNA
- 0.5 µl forward Primer
- 0.5 µl reverse Primer
- 5 µl Hotstar Taq Mastermix (Quiagen, Netherlands)
- 2 µl H_20

Reaction volume: 10 µl

Amplification was conducted using following cycling conditions:

Temperature in °C	**Time**	**Note**
94	15 min	
94 57 72	30 sec 30 sec 45 sec	34 cycles
72	7	
8	∞	

PCR products were checked by gel electrophoresis at 130 V in 1% agarose G´gel (0.7 g LE-Agarose, 70 ml TBE Puffer, stained with 7 µl GelRedTM [Biotium, US]).

Sequencing reaction was set up twice for each PCR-product, once with forward and once with reverse primer:

- 0.5 µl PCR-product
- 0.3 µl Primer
- 0.5 µl BigDyeTM Terminator v3.1 (Applied Biosystems, US)
- 1.4 µl 5X Sequencing Buffer (Applied Biosystems, US)
- 7 µl H_20

Reaction volume: 10 µl

Sequencing reaction was conducted using following cycling conditions:

Temperature in °C	**Time**	**Note**
96 50 60	30 sec 30 sec 45 sec	24 cycles
4	∞	

Sequencing products were filtered using soaked Sephadex G-50 Superfine (GE Healthcare – Life Sciences, US) and Centri-SEP columns (Invitrogen, US) to remove remaining sequencing reagents. Columns filled with soaked Sephadex were centrifuged for 5 sec and again for 2 min at 750 rcf, removing excess water after both centrifugation steps. Sequencing

products were diluted 1:1 with RNase free H_2O and pipetted onto separate columns, which were then centrifuged at 750 rcf for 2 min.

The filtered samples were sequenced on ABI 3130 xl ABI Prism (Applied Biosystems, US). Sequences chromatograms were visualized with 4Peaks (available at https://nucleobytes.com/4peaks/index.html) and compared against the reference sequence (GRCh37/hg19) on UCSC genome browser (http://genome.ucsc.edu).[104]

3 Results

3.1 Clinical outcome

On overview of the clinical symptoms is given in Table 3, while Figure 5 shows photographs of both individuals.

	Individual	BD9-1	OPH22-4
General characteris	Age	~13	~24
	Gender	Male	Male
	Age of onset	Congenital	Congenital
	Height	155 cm	152 cm
	Occipitofrontal circumference	53 cm	52 cm
Major criteria	BMI	27 (overweight)	27,9 (overweight)
	Polydactyly and syndactyly	Hexadactyly of both feet, synhexadactyly of both hands	Hexadactyly of both feet and right hand, synheptadactyly of left hand
	Intellectual disability	Mild	Severe
	Retinitis pigmentosa	Yes	Yes
Minor criteria	Strabismus	No	No
	Nystagmus	No	Yes
	Color blindness	No	No
	Eyesight	Weak	Weak
	Communicative abilities	Weak	Nil
	Behavioral expression	Hyperactive	Lethargic
	Attention	Yes	No
	Deafness	No	No
	Renal function test	Normal	Borderline
	Cardiac Status	Normal	Normal
	Liver function status	Abnormal (high alanine aminotransferase and alkaline phosphatase)	Abnormal (high alanine aminotransferase and alkaline phosphatase)
	Lipid profile	High (abnormal triglycerides)	Normal
	Diabetes	No	No
	Digestive system functionality	Normal	Normal
	Facial morphology	Normal	Normal
	Dental anomalies	No	No
	Growth condition	Normal	Weak
	Muscle degeneration	No	No
	Abnormal spine curvature	No	No
	Gait	Wide	Wide

Table 3: Clinical symptoms of affected individuals BD9-1 and OPH22-4. General characteristics are listed in the first part, major diagnostic criteria in the second and minor diagnostic criteria in the third part of the table. Of the major criteria, renal anomalies and hypogonadism was not assessed.[103]

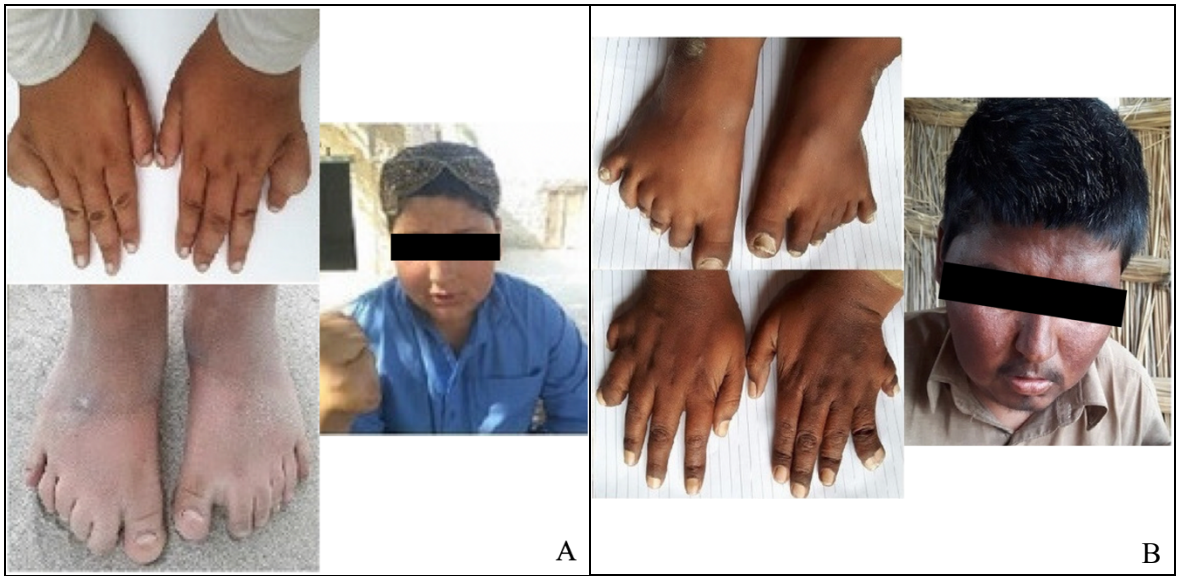

Figure 5: Photographs of affected individuals. A. Individual BD9-1 with synhexadactyly of both hands and hexadactyly of both feet. **B. Individual OPH22-4** with synheptadactyly of left hand and hexadactyly of right hand and both feet. **A+B.** Absence of typical facial features (hypoplasia of the zygomatic bone, retrognathia, depressed nasal bridge and deep set eyes)[52] in both individuals.[103]

Both individuals were diagnosed with BBS. They exhibit four major criteria (obesity, polydactyly, intellectual disability and retinitis pigmentosa), thus fulfilling the diagnostic criteria. Hypogonadism and structural renal anomalies were not assessed in both individuals.

In comparison, BD9-1 was less severely affected with mild intellectual disabilities, delayed speech development and hyperactive behavioral expression. Renal function was normal, as well as cardiac status and digestive system functionality. Liver function was abnormal with high levels of alanine aminotransferase and alkaline phosphatase, and triglyceride levels were elevated.

OPH22-4 was more severely affected with severe intellectual disabilities. He had developed no communication abilities and had a lethargic behavioral expression. Renal function was borderline, liver function abnormal with high alanine aminotransferase and alkaline phosphatase levels, and lipid profile was normal.

3.2 Marker analysis

3.2.1 Family BD9

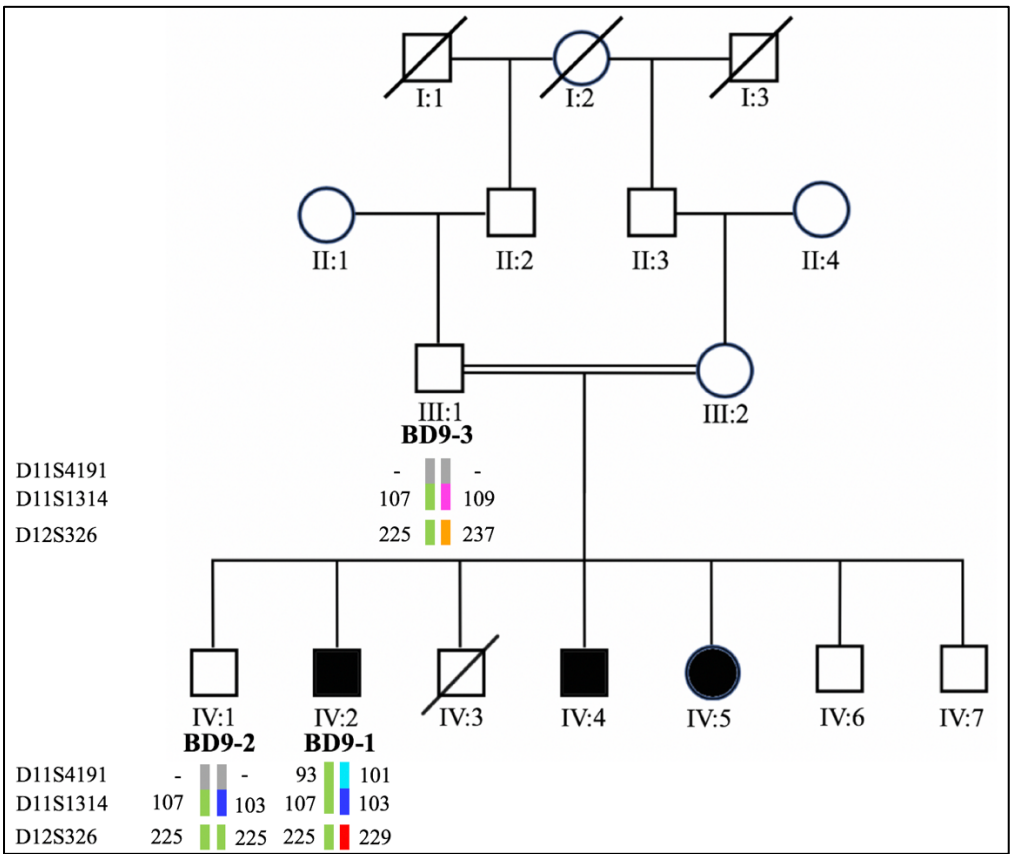

Figure 6: Pedigree of family BD9 with microsatellite allele sizes. Markers D11S4191 and D11S1314 on chromosome 11 surrounding *BBS1* and marker D12S326 on chromosome 12 next to *BBS10*. No marker was found homozygous in the affected individual. Grey bars indicate were no result could be obtained. (Created in PowerPoint)

In the affected individual BD9-1, no markers were found to be identical in allele size. For the non-affected individuals, results could not be obtained for marker D11S4191 due to a pipetting error. However, the analysis was not repeated as the results render identity by descent for *BBS1* and *BBS10* unlikely in BD9-1.

3.2.2 Family OPH22

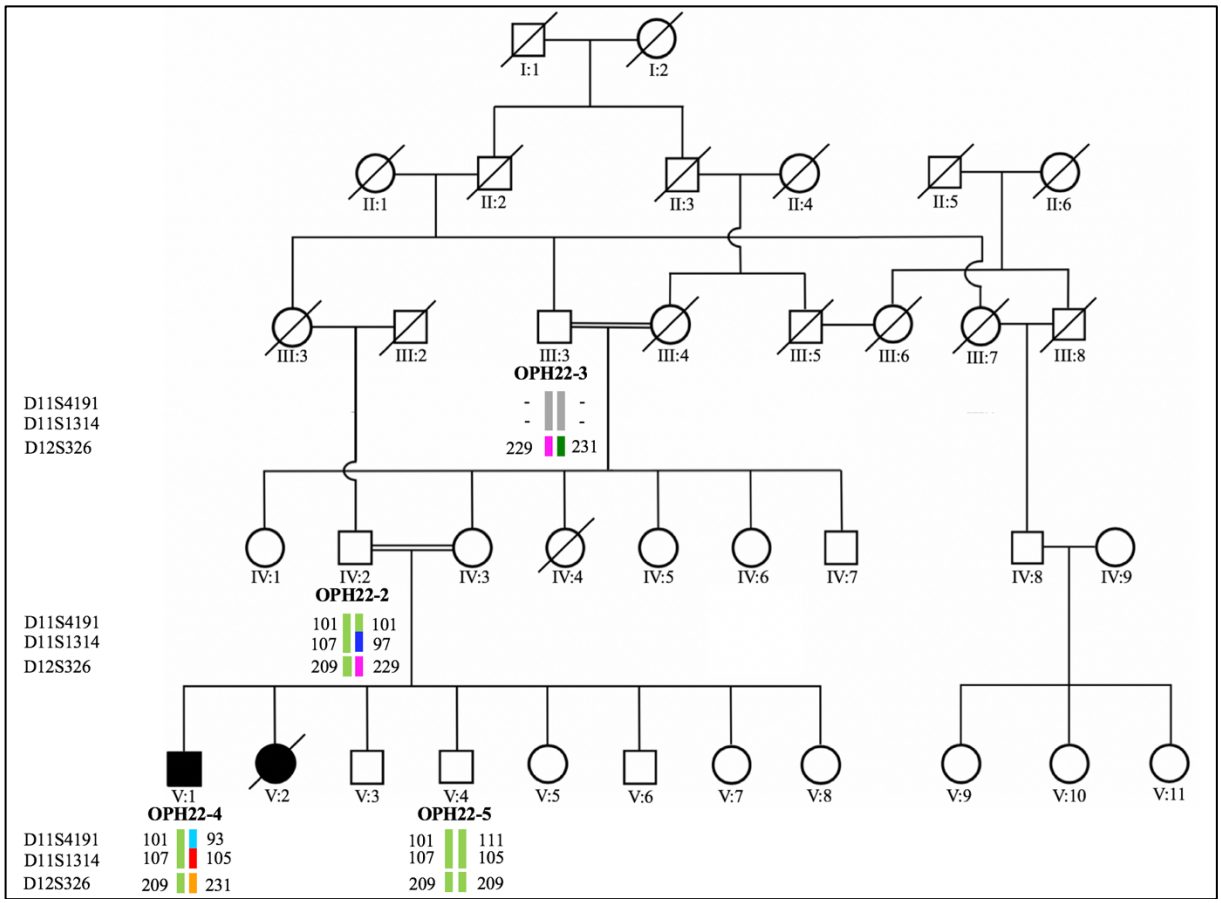

Figure 7: Pedigree of family OPH22 with microsatellite allele sizes. Markers D11S4191 and D11S1314 on chromosome 11 surrounding *BBS1* and marker D12S326 on chromosome 12 next to *BBS10*. No marker was found homozygous in the affected individual. Grey bars (-) indicate that no result could be obtained. (Created in PowerPoint)

In the affected individual OPH22-4, no markers were found to be identical in allele size. For the non-affected individual OPH22-3, results could not be obtained for marker D11S4191 and D11S1314 due to a pipetting error. Again, the analysis was not repeated as the results render identity by descent of *BBS1* and *BBS10* unlikely in OPH22-4.

As *BBS1* and *BBD10* could be ruled as unlikely causing BBS in the two individuals, whole exome sequencing was conducted next to determine the sequence and homozygosity of all known BBS genes.

3.3 Whole exome sequencing

3.3.1 Individual BD9-1

Whole exome sequencing of affected individual BD9-1 revealed 38605 variants in total. 48 were located in known BBS genes, of which 18 were located in exons. After filtering out synonymous variants, 10 missense and frameshift variants remained. Only two variants exhibited a frequency of less than 1%. None of the two variants could be ruled out on the basis of protein prediction software. Filtering steps are visualized in Figure 8.

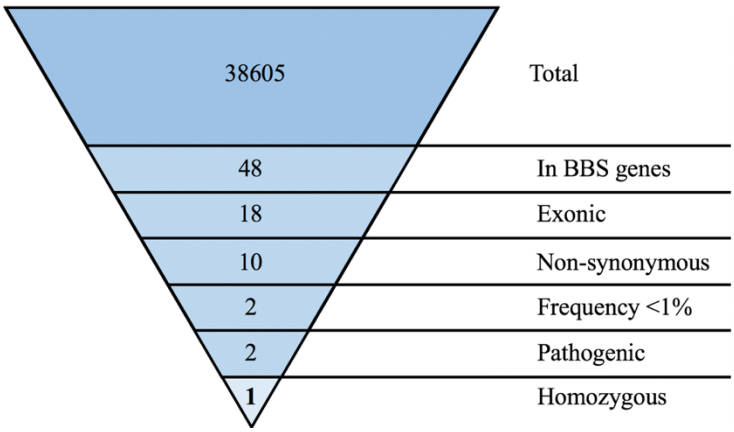

Figure 8. Funnel chart of number of variants after each filtering step. (BD9-1, created in PowerPoint)

The first variant is located in ***IFT172/BBS20:*** NM_015662:c.C4990T:p.R1664W. It is a single base substitution in exon 46 of Cytosine for Tyrosine, resulting in an amino acid change from Arginine to Tryptosine. This variant is listed in dbSNP (rs139348179)[108] and in GnomAD, where it is reported with an allele frequency of 0.2102% in the global population and of 1.850% in the South Asian population (https://gnomad.broadinstitute.org/).[109] In silico programs predict a possibly damaging (PolyPhen-2 HumVar Score 0.771) or a tolerating (SIFT Score 0.188) effect on protein function.

The second is located in ***BBS9***: NM_198428:c.299delC:p.S100fs. It is a single base deletion of Cytosine in exon 4, disrupting the reading frame and converting codon 123 into a stop codon, resulting in a prematurely terminated polypeptide of 122 amino acids. This variant has been previously published by Muzammil Ahmad Khan et al. and was found in two BBS patients of a consanguineous family recruited from the same region (Dera Ismail Khan

district of Khyber-Pakhtoonkhwa Province) of Pakistan. Upon identification, the variant was absent from various databases and was not found in 100 Pakistani and Arab controls.[110]

As only the frameshift mutation in *BBS9* was found homozygous, this variant was selected for segregation analysis.

3.3.2 Individual OPH22-4

Whole exome sequencing of affected individual OPH22-4 revealed 38074 variants in total. 46 were located in known BBS genes, of which 19 were located in exons. After filtering out synonymous variants, 11 missense and frameshift variants remained. Only one variant exhibited a frequency of less than 1%. This variant was the identical frameshift deletion in *BBS9* (NM_001033604:c.299delC:p.S100fs) which was also found in individual BD9-1. As it was homozygous, it was selected for segregation analysis. Filtering steps are visualized in Figure 9.

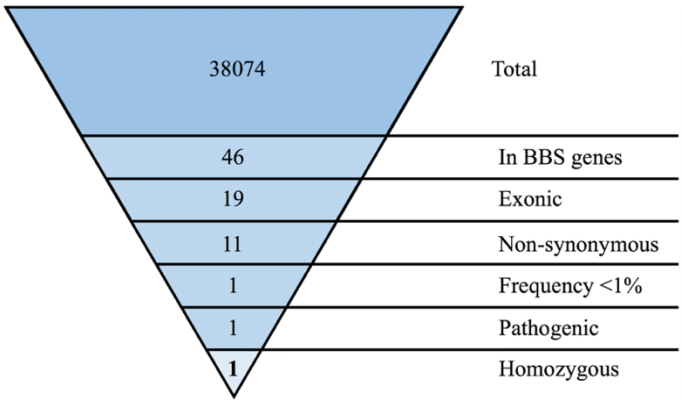

Figure 9: Funnel chart of numbers of variants after each filtering step. (OPH22-4, created in PowerPoint)

3.3.3 Homozygosity mapping

Runs of homozygosity over the genome were mapped and compared with BBS gene loci. In **BD9-1,** *BBS9* was the only BBS gene to be located within a ROH, which was 14 Mbp in size. This corroborates the deletion in *BBS9* as the candidate variant.

In **OPH22-4,** *BBS9* and *NPHP1/BBS22* loci were found to overlap with ROHs. *BBS9* was located within a ROH of 7 MBp in size, and *NPHP1/BBS22* within a ROH of 4 Mbp. As no variants were found in *NPHP1/BBS22*, *BBS9* remains as the most likely gene to cause BBS in this individual.

As ROH overlapped between both affected individuals, genotypes were compared manually and revealed an identical haplotype of over 7 Mbp harboring the candidate variant, thus suggesting a relation between both families.

Figure 10 shows ROH encompassing *BBS9* in both individuals and *NPHP1/BBS22* in individual OPH22-4.

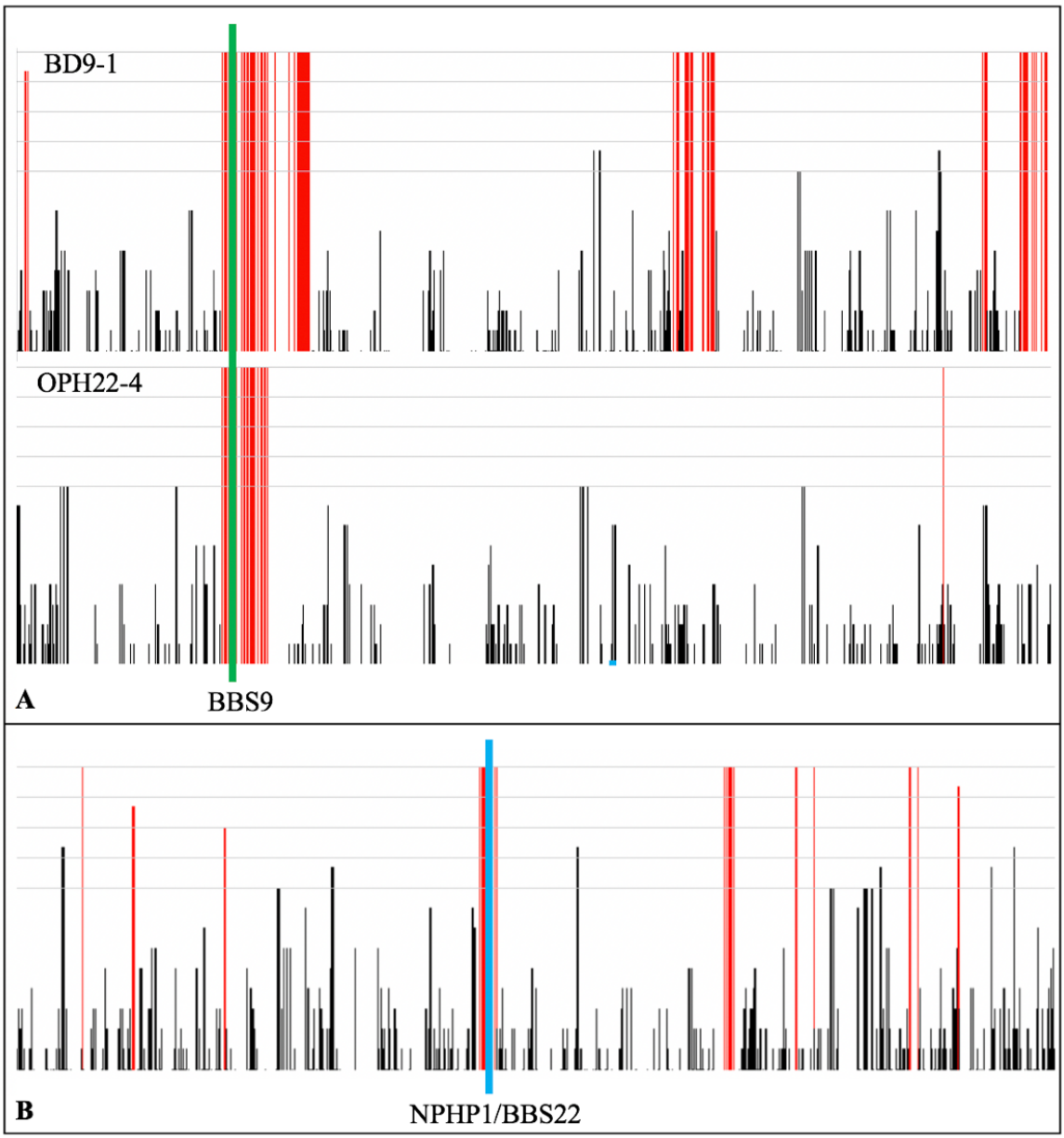

Figure 10: Runs of homozygosity overlapping BBS loci. A: Runs of homozygosity mapped on chromosome 7 of individual BD9-1 (above) and OPH22-4 (below). Red bars indicate runs of homozygosity, **green** bar indicate *BBS9* locus. **B. Runs of homozygosity mapped on chromosome 2 of individual OPH22-4. Red** bars indicate runs of homozygosity, **blue** bar indicates *NPHP1/BBS22* locus. (Picture created with HomozygosityMapper, annotated in PowerPoint.)

Figure 11 gives an overview of chromosome 7 with *BBS9* locus, homozygous regions of affected individuals, *BBS9* exon-intron structure and the sequence affected by the c.299delC variant.

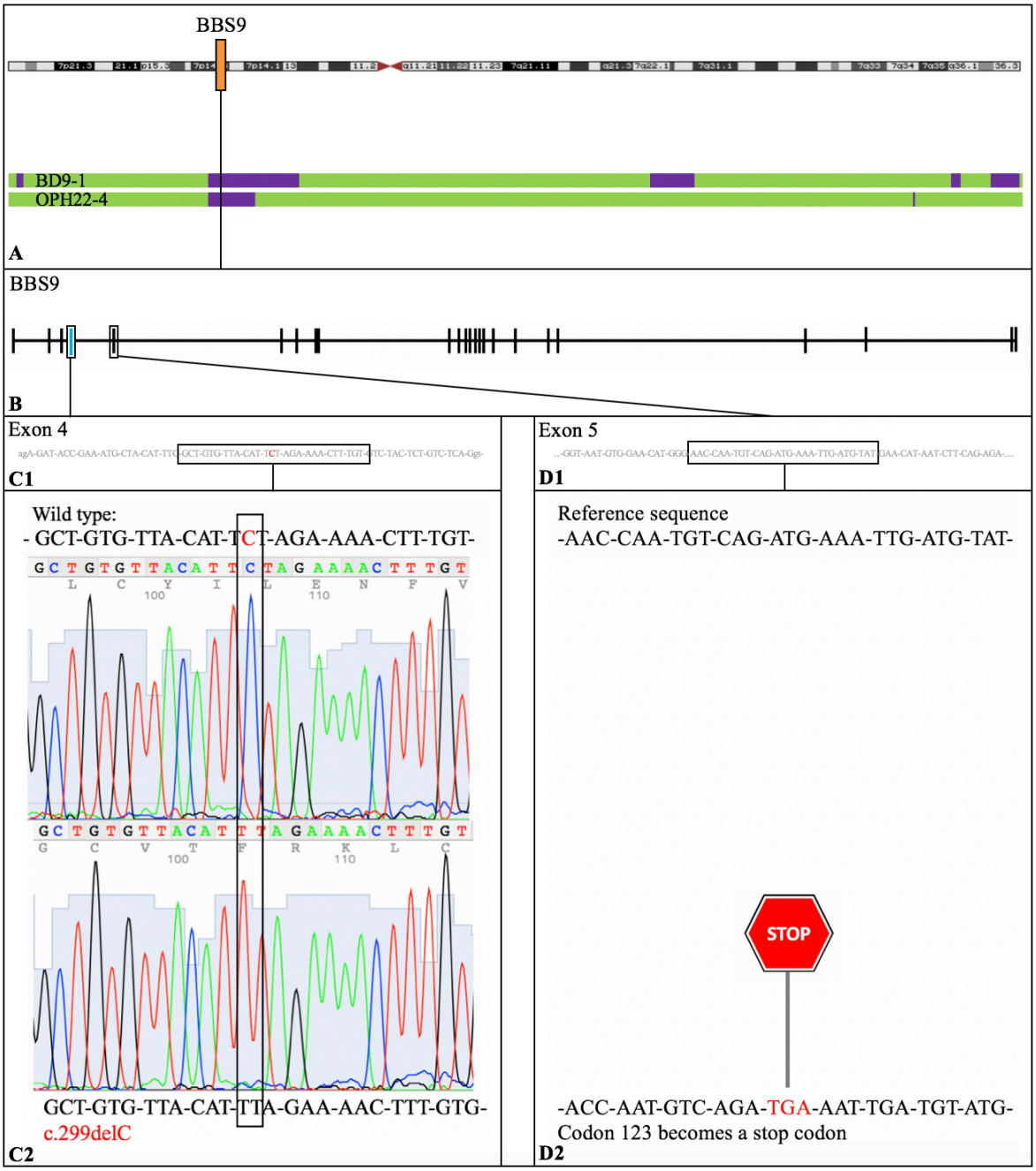

Figure 11: From chromosome 7 to a single base deletion. A. Ideogram of chromosome 7 with BBS9 locus (**orange** bar) above, runs of homozygosity mapped on chromosome 7 (**violet** regions) of individual BD9-1 and OPH22-4 below. **B. Exon intron structure of BBS9**, exon 4 marked in **blue**. **C1. Reference sequence of exon 4. C2. Sequence chromatogram of exon 4 (from base 286 to 312).** Wild type (above), homozygous deletion of cytosine at position 299 in an affected individual (below). **D1. Partial reference sequence of exon 5. D2 Sequence of exon five (from base 355 to 381).** Wild type (above), shift of reading frame leading to the emergence a premature stop codon at codon number 123 (below). (Picture created with USCS genome browser, 4Peaks and PowerPoint)

3.4 Sanger sequencing

Exon 4 of *BBS9* was sequenced by Sanger Sequencing in all available family members, confirming the homozygous deletion c.299delC in both affected individuals. The same mutation in a heterozygous state was in the fathers of both individuals and in the maternal grandfather of individual OPH22-4. The unaffected brother of BD9-1 was no carrier of the mutation, while the unaffected brother of OPH22-4 was a carrier.

3.4.1 Segregation analysis of family BD9

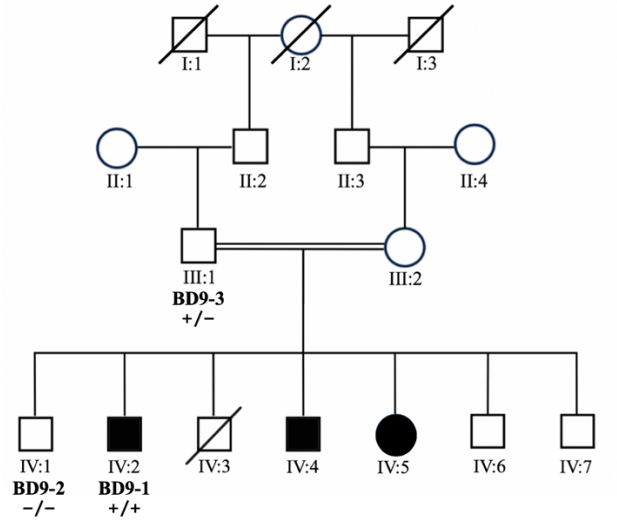

Figure 12: Segregation of c.del299C in family BD9. +/+ indicates the deletion in homozygous state, +/- in heterozygous state (carrier) and -/- indicates homozygous wildtype. C.del299C segregated with disease in family BD9. (Picture created in PowerPoint)

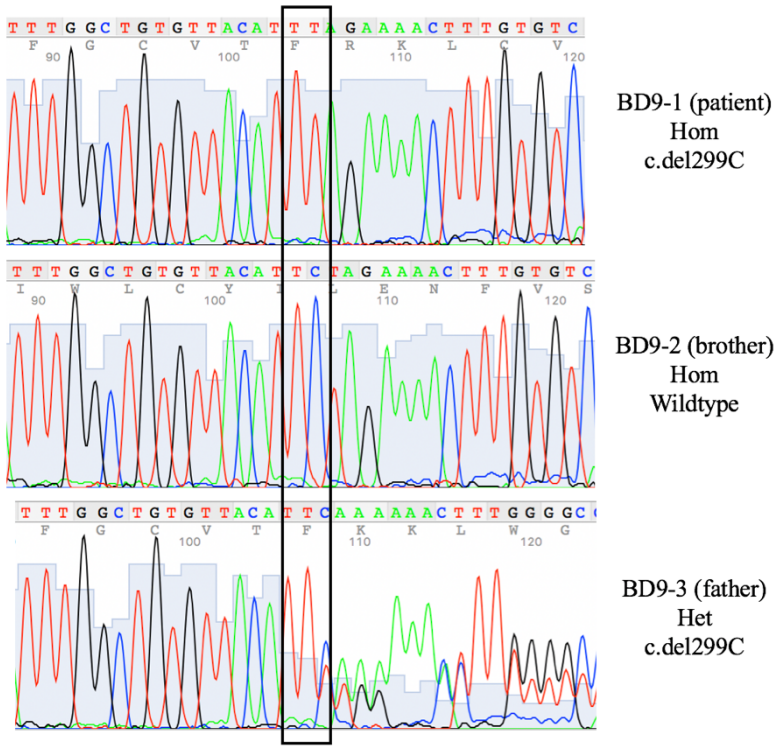

Figure 13: Sequence chromatographs of available family members of BD9. The father of the affected individual was found to carry the mutation c.del299C heterozygous, the affected individual homozygous, while the unaffected brother was found to by homozygous for the wildtype allele. (Picture created with 4Peaks and annotated in PowerPoint)

3.4.2 Segregation analysis of family OPH22

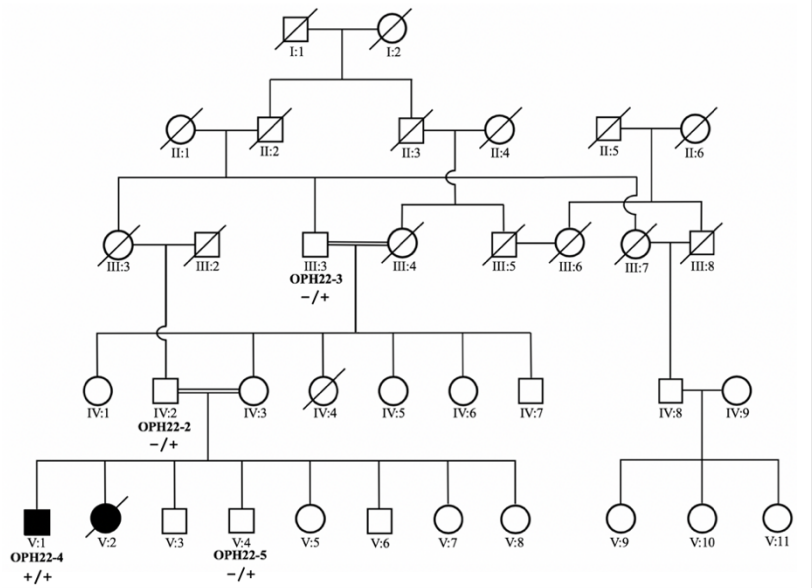

Figure 14: Segregation of c.del299C in family OPH22. +/+ indicates the deletion in homozygous state, +/- in heterozygous state (carrier) and -/- indicates homozygous wildtype. C.del299C segregated with disease in family OPH22. (Picture created in PowerPoint)

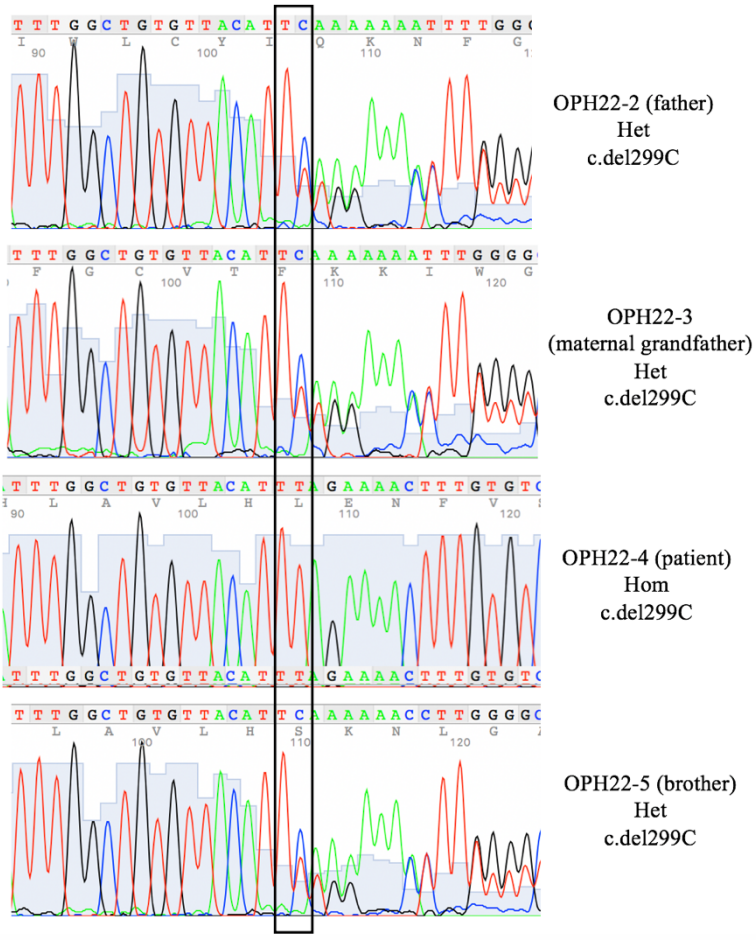

Figure 15: Sequence chromatographs of available family members of OPH22. Brother, father and maternal grandfather of the affected individual were carriers of the mutation c.del299C, while it was found homozygous in the affected individual. (Picture created with 4Peaks and annotated in PowerPoint)

3.4.3 Intronic insertion

Sanger sequencing with the first primer pair (BBS9E4forward and BBS9E4reverse) encompassing 255 bp of exon 4 as well as 79 bp upstream and 22 bp downstream revealed an intronic insertion at the position NM_198428.3:c.328+15_328+16, beginning with the sequence TTTTCTTTTC(T)$_n$. The complete inserted sequence could not be obtained, likely because of polymerase slippage due to repeats of homopolymers in the inserted sequence (Figure 16). Electrophoresis of the PCR amplicons suggest an insertion length of approximately 300 bp. Similar insertions for the same locus can be found in dbSNP (rs775674614), starting with the same sequence but only 32 to 36 nucleotides in length.[111]

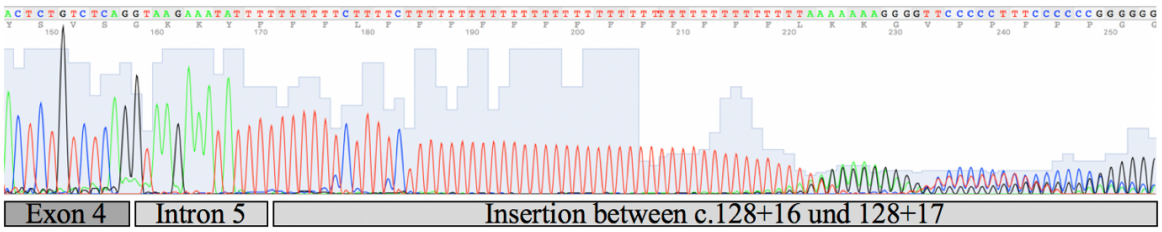

Figure 16: Sequence chromatograph of individual BD9-1. 12 nucleotides of exon 4 are pictured, along with 16 of intron 5. Between 16 and 17 nucleotides downstream of the last coding one, the inserted sequence starts with TTTTTCTTTTC(T)n and soon becomes unreadable. Exon 4, intron 5 and inserted sequence are marked with grey bars. (Sequence chromatogram visualized with 4Peaks and annotated in PowerPoint.)

This insertion was found homozygous in both affected individuals. In the individuals heterozygous for the c.299delC mutation, comparison between first sequencing reaction and second sequencing reaction (with primers BBS9_SB_f and BBS9_SB_r) confirmed allelic drop-out for either the mutant allele or the wild type allele. In the three individuals homozygous for either allele, no allelic drop-out occurred. Thus, segregation of the mutant allele with the intronic insertion is likely, leading to allelic drop-out due to different amplicon sizes in heterozygous individuals.

3.5 Founder effect

From initial pedigree assessment, the families described in this study were not related. However, results from exome sequencing identified a shared haplotype of approximately 7 Mbp in both affected individuals, suggesting a common ancestor. Therefore, both families of this current study as well as the family from the previous study, in which the c.del299C mutation was first described, were revisited. While the families from this study belong to the Rehmani-khail tribe, the third family were recruited from a neighboring village and belonged to the Khano-khail tribe. Exploring the ancestral history with the families' elders, a common ancestry with the Khaisoori tribe seems possible.[103]

4 Discussion

4.1 BBS9

The *BBS9* gene is located on the forward strand of chromosome 7. It consists of 30 exons and 14 isoforms are produced by alternate splicing.[112] The canonical isoform (encoded by transcript version 2) consists of 887 amino acids and has a molecular weight of 99.280 Kilodalton (Uniprot Q3SYG4)[113]. It is expressed in various adult human tissues, including the heart, brain, placenta, skeletal muscle and kidney, as well as in fetal tissues.[114]

Initially, the protein was discovered in the cellular response of an osteoblast-like osteosarcoma cell line to Parathyroid hormone (PTH) stimulation.[114] As its expression was downregulated by PTH stimulus, it is also known as Parathyroid hormone responsive protein B1 (PTHB1).[115] Veleri et al. identified *PTHB1* to be interrupted by a chromosomal translocation associated with Wilms tumor.[116] In 2005, Nishimura et al. identified the *[PTH]B1* gene as the 9th BBS gene by combining homozygosity mapping of small consanguineous BBS families with gene expression studies and comparative genomics. In this study, 6.3% of unrelated BBS patients carried a homozygous *BBS9* mutation.[32]

The BBS9 protein has been identified as a component of the BBSome, but its molecular function is still mostly unknown. Of its tertiary structure, only the N-terminal domain has been studied experimentally, revealing a seven-bladed ß-propeller comprised of amino acids 1 to 407, shown in Figure 17. This structural element is shared with three other BBSome proteins (BBS1, BBS2, BBS7) and other proteins involved in forming membrane coats for intracellular vesicle transport.[117]

Figure 17: Seven-bladed ß-propeller comprised of amino acids 7-407 of BBS9, pictured with Mol* 3D viewer at RCSB PDB (PDB ID: 4YD8).[117]

The other tertiary structural elements are predicted to be a beta sandwich domain of the immunoglobulin type and an alpha/beta platform, both shared with BBS2 and BBS7.[70] At the C-terminus of BBS9, amino acids 685 to 785 have been identified as the binding site for LZTFL1/BBS17. This protein regulates ciliary trafficking by interacting with the BBSome through BBS9.[34]

4.2 *BBS9* variants

The Human Gene Mutation Database (HGMD, http://www.hgmd.cf.ac.uk/ac/index.php)[118] lists 48 variants associated with disease in *BBS9*, excluding gross deletions and complex rearrangement. Of 48 variants, 39 are associated with BBS phenotype. Other associated phenotypes are obesity (1), retinitis pigmentosa (1), macular dystrophy (2) and nonsyndromic craniosynostosis (1).

Of the 39 BBS variants associated with BBS, 16 were missense, 10 nonsense, nine splice variants, five deletions and two insertions.

Notably, only missense variants are associated with non-BBS phenotypes (i.e. retinitis pigmentosa, macular dystrophy and nonsyndromic craniosynostosis), except one frameshift deletion c.T310del (p.Cys104Valfs*20). This deletion was found in one 14 year old obese patient compound heterozygous with the missense mutation c.2258A>T (p.Glu753Val)[119] and is located 11 base pairs downstream of the deletion described in this study. Both result in frameshift and are predicted to convert codon 123 into a stop codon. The deletion c.T310del has not been found homozygous or associated with BBS phenotype so far. It has been reported with an allele frequency of 0.00003 in GnomAD (https://gnomad.broadinstitute.org/).[109]

The c.del299C (p.Ser100Leufs*24) deletion found in this study is located in codon 100, where it results in a change from amino acid Serine to Leucine. The shift of the reading frame is predicted to result in the premature termination of the polypeptide at amino acid 123. Figure 18 shows amino acid sequence that is expressed normally highlighted in light green. Exon 4, in which the deletion occurs, is contained in all isoforms except isoforms 5, 12 and 13.

However, the possibility that the intronic insertion found in both affected individuals may affect either expression or splicing of the protein cannot be excluded.

Figure 18: Effect of c.299delC (p.Ser299Leufs*24) on BBS9. Seven-bladed ß-propeller comprised of amino acids 4-407 of BBS9 pictured with 3D viewer of RSCB PDB (PDB ID: 4YD8).[117] Amino acids 3 to 99 that are not affected by the frameshift resulting from c299delC (p.Ser299Leufs*24) are highlighted in **light green**.

4.3 Genotype-phenotype correlation

The c.del299C variant has been found homozygous to date in 6 BBS patients; two patients from the original family published in 2016[110], two patients of this study and two further affected siblings of individual BD9-1 that were not included in this thesis, but later sequenced for the variant.[103]

Of the major features, retinitis pigmentosa, obesity and polydactyly have the highest reported prevalence of 93–100%, 72–92% and 63–81% respectively.[5,120] In these six patients, retinitis pigmentosa has been observed in all patients except the youngest, who was eight years old when examined. Obesity was present in all six patients, as well as polydactyly of all four limbs.[103, 110]

Renal anomalies, hypogonadism and development delay are less prevalent in BBS patients and also very variable in their manifestation.[5,120] Hypogonadism was assessed in only 2 patients where it was absent, but one female patient had irregular menstrual cycles. Renal function ranged from normal to abnormal, but renal structure was not assessed. Intellectual disability was present in all four patients in this study with varying severity, as three patients from family BD9 had mild intellectual disability and weak to normal communication abilities, while the patient from family OPH22 had severe intellectual disability and had not developed any communication abilities at the age of 24. [103, 110]

Additional symptoms were variable as well, including strabism, hearing loss, abnormal lipid and liver profile, digestive problems, attention deficit and an abnormal gait.[103, 110]

This far from uniform phenotypical presentation in six patients with the same genotype illustrates the phenotypical heterogeneity of BBS.

Establishing genotype-phenotype correlation for individual BBS genes has been attempted, which would improve molecular diagnostics, enable a targeted disease management as well as allow prognosis about patients' development.[121] Apart from the clinical implementation, such correlations could elucidate the role of individual BBS proteins in cilia physiology and disease developement.[36] Small sample sizes, differences in the clinical assessment of patients and the genetic heterogeneity of the syndrome have rendered it so far impossible to establish distinct genotype-phenotype correlations.[121] However, some studies have analyzed the primary BBS symptoms or a subset thereof in patient cohorts and observed trends for individual genes:

In 2015, Castro-Sanchez et al. studied *BBS1* and the chaperone complex genes *BBS6*, *BBS10* and *BBS12* and found that mutations in the chaperone genes lead to a more severe phenotype with a higher prevalence of all primary features, but especially of cognitive impairment in *BBS12* and urogenital anomalies in *BBS10*. In *BBS1* mutations, polydactyly was seen less frequent than in chaperonin proteins, and only sporadically all four limbs were affected. In patients with the recurrent *BBS1* mutation Met390Arg, a later onset of visual symptoms was observed.[121]

In 2017, Forsythe et al. studied the genotype-phenotype correlation of renal disease in 265 patients and found that mutations in *BBS2*, *BBS0* and *BBS12* were associated with more severe renal disease than *BBS1*. Additionally, homozygous or compound heterozygous protein truncating mutations were also associated with more severe renal disease then homozygous missense mutations.[16]

In 2010, Hjortshøj et al. found similar genotype-phenotype correlations regarding the milder phenotype of *BBS1*.[122]

BBS9 was found mutated in these studies either at low frequencies[16], was not included in the study design[121] or has not yet been identified when the study was conducted.[122] On account of this, no *BBS9* genotype-phenotype correlations were established by these above mentioned cohort studies.

In 2019, Niederlova et al. published a meta-analysis based on 85 published studies analyzing the variability of the primary BBS symptoms. Patients were grouped according to the functional class of the mutated gene (BBSome, chaperone complex, *BBS3*) and the presumed severity of the mutation (partial versus complete loss of function). Based on 426 patients for whom presence or absence of five primary features (excluding hypogonadism) was reported, severity of the syndrome (how many primary symptoms were present) and penetrance of individual symptoms were analyzed. In this set, patients with mutations in *BBS3* had the lowest disease severity, followed by *BBS1* and *BBS8*. Patients with *BBS3* mutations had lower penetrance of cognitive impairment and renal anomalies than patients with mutations in other genes. Of mutations in the BBSome subunits, *BBS2* had the highest penetrance of polydactyly, and *BBS1* the lowest. For renal disease, high penetrance was observed in *BBS2*, *BBS7* and *BBS9* (>60%), while low penetrance was observed in *BBS1*, *BBS4* and *BBS8* (>30%). For other symptoms, no conclusive statistical analysis could be carried out.

Due to its high prevalence, *BBS1* and in particular the Met390Arg missense mutation, is the best studied. However, even the clinical presentation of this mutation is far from uniform, ranging from homozygous individuals who do not show any symptoms of BBS[37] to patients exhibiting the classic BBS phenotype with various secondary symptoms.[123] This demonstrates the difficulty of establishing a distinct genotype-phenotype correlation even for the most frequent mutation. Taking into account that many BBS mutations are private mutations found only in a single family[35, 124], it is hardly surprising that no distinct correlation between genotype and phenotype has been found. Triallelism has been proposed to account for the heterogeneity of the phenotype[63], but while a third possible pathogenic allele has been found in cohorts more frequently than it would be expected based on carrier frequencies, the correlation of the third allele with a more severe phenotype has not been proven strong enough to consider BBS something other than an autosomal recessive disease.[35, 125, 126] Considering that there has even been male monozygotic twins born with BBS, one of which had three additional digits at birth, while the other had none,[5] the answer to the variability of the phenotype might not be found in the genetic differences after all.

4.4 BBS as a model ciliopathy

Before the discovery that defects in the primary cilium lead to the diverse group of disorders known today as ciliopathies, each disorder was considered a rare yet distinct clinical entity, named after the clinicians who had first described it. Most syndromes are a unique

combination of symptoms in a number of organs, but the organ manifestation itself are not unique to each syndrome.[127]

Retinitis pigmentosa (RP) alongside cone-dystrophy, cone-rod dystrophy and macular dystrophy form the group of non-syndromic retinal ciliopathies, affecting only a single organ.[128] Nephronophthisis (NPHP) and the polycystic kidney diseases are renal ciliopathies.[129,130] Both retinal dystrophy and NPHP can occur together, then called Senior-Loken Syndrome (SLS, MIM: 266900). BBS and Usher syndrome (RP and congenital deafness, MIM: 276900) are the most common syndromic causes for RP.[75]

Polydactyly is another frequent feature of ciliopathies, occurring in Jeune's asphyxiating dystrophy (JATD, MIM: 208500), Meckel-Gruber syndrome (MKS, MIM: 249000) and Joubert syndrome (JS, MIM: 213300). Obesity and Diabetes mellitus type 2 are seen in Alström syndrome (ALMS, MIM 203800). Abnormalities of the central nervous system in the form of hypoplasia of the cerebellar vermix are seen in Joubert-Syndrome (JBTS, MIM: 213300) and in the form of occipital encephalocele in Meckel-Gruber syndrome. Less frequently observed in BBS are liver fibrosis (seen in Joubert and Jeune's syndrome), hydropmetrocolpos (seen in McKusick-Kaufman syndrome) and hearing impairment (seen in Alström syndrome).[75]

This list demonstrates the high pleiotropy exhibited by ciliopathies, meaning that one mutated gene causes symptoms in a variety of organs.[75] It also illustrates that each individual symptom can have many different genetic causes. What is more, when the first disease genes were being identified for a specific disorder, they were often found as the cause for a different disorder as well.

This allelic variability is demonstrated in *BBS6,* the first BBS gene that was identified in September 2000.[30] It had been identified as *MKKS* causing McKusick-Kaufman syndrome in May of the same year.[131] The broadest disease spectrum by far has been found to date for the centrosomal protein *CEP290,* which is also known as *BBS14* and *NPHP6.* It ranges from single organ Leber congenital amaurosis and Nephronophthisis over multi system disorders like Senior-Loken syndrome, Jeune syndrome and Bardet-Biedl syndrome to the most severe, perinatally lethal, Meckel-Gruber Syndrome.[75, 47]

In some cases, specific mutations are limited to a single phenotype: A splice-mutation in *CEP290* has been only associated in LCA patients, which is found in up to a fourth of patients in some populations.[132] In one Pakistani family, a *BBS8* mutation was identified to

cause isolated RP, as the mutation affected only a retina specific isoform produced by alternate splicing.[133] In other cases, the severity and location of the mutation was found to correlate with the severity of the phenotype, as hypomorphic *NPHP3* alleles have been implicated in NPHP, while null alleles in the same gene cause Meckel-Gruber syndrome. Similarly, hypomorphic *MKS1* alleles were found in BBS, while null alleles were found in MKS. In most cases however, such genotype-phenotype predictions are not possible:[134] In *CEP290* alone, more than twenty mutations segregating with two or three phenotypes have been identified.[132]

As the genotype at the primary disease locus cannot sufficiently explain this variability, the focus has shifted to studying ciliopathies in the context of the complex ciliary network. Several modifying interactions between individual ciliopathy genes have been documented.[134]

In this context, the model of epistatic interactions between a primary disease driver, who in itself is sufficient to cause the onset of a disorder, and second-site modifiers who exacerbate or alleviate the phenotype, has been introduced. The modifying alleles are variants not sufficient to cause the phenotype, or heterozygous pathogenic alleles in other ciliopathy genes or variants in genes that do not cause a ciliopathy itself but interact with the mutated gene in the network of the cilium.[134, 135, 136]

Similarly, instead of specific modifiers, it has been proposed that the mutational load of the whole ciliary proteome has the potential to modify the phenotype. This is the total number of variants in genes coding for the ciliary proteome, their quality and locus. The variants may be pathogenic, protective or common variants, and their contribution taken by itself may be small, but through complex interactions they are able to modify the severity and penetrance of the phenotype.[55, 134]

When each disorder is considered not as an isolated entity but within the spectrum of the ciliopathies, the complexity of the cilium can be taken into consideration and more studies are able to be conducted. As each ciliopathy is individually rare, patients potentially participating in such studies are scarce. Taken together, however, ciliopathies exceed a frequency of 1:1000 in live births, thus justifying extensive research into the disease mechanisms, diagnostic tools and the development of a targeted treatment.[137]

4.5 Summary

In this study, the molecular basis of BBS in two Pakistani families has been explored. In both affected individuals, a frameshift deletion in *BBS9* has been identified as causal for the disorder. This variant has been found once in a BBS family recruited from the same region (Dera Ismail Khan district) in Pakistan. No relation between the three families was suspected on initial pedigree exploration, however, as both affected individuals shared a haplotype harboring the mutation, a common ancestor was suggested. Upon revisiting family elders, a possibly relation with the Khaisoori tribe was established for all three families, suggesting a common founder for the disease allele.

These results offer the affected families a molecular confirmation of the clinical diagnosis and establish the possibility of carrier testing for the extended family. If desired, marriage choices can be made based on the carrier status of the familial BBS mutation.

This report of the clinical presentation of two affected BBS patients also extends the phenotype of the disorder. Comparisons with other *BBS9* phenotypes are difficult to establish, firstly because *BBS9* patients in larger cohort studies are scarce, and secondly because BBS, like other ciliopathies, evades the establishment of distinct genotype-phenotype correlations because of its genotypic and phenotypic variability as well as its oligogenic properties.

Nevertheless, identification of variants causal in BBS patients extends the repertoire of disease-causing variants and supports defining the pathogenicity of novel variants. Defining the mutational spectrum of the Pakistani population enables for a targeted molecular diagnosis of affected individuals, especially if a founder mutation is suspected.

Characterization of disease-causing variants contributes to the larger goal of understanding the workings of the cilium and development of disease associated with a malfunction of this cell organelle. This in turn paves the way to offer best clinical management and develop targeted treatments for patients suffering from BBS or another ciliopathy.

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