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Connections between genetic spectrum and clinical picture in ciliopathies

SUMMARY OF PhD THESIS

PhD coordinator:
PROF. UNIV. DR. MIHAELA BĂLGRĂDEAN

PhD candidate:
INA OFELIA FOCȘA

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D-nei Dr. Erica Ellen Davis

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Content

INTRODUCTION

This work aims to approach a relatively recent defined field: the ciliopathies, whose swiftly evolution leads to the development of oligogenic and triallelic inheritance concepts. Ciliopathies are a large category of disorders with heterogenous clinical picture, and multiorgan involvement whose genetic substratum is responsible for structural or functional impairment of cilium. Each clinical entities of this group of disorders are, actually, a rare disease with incidence varying between 1:50.000 to 1:150.000. However, collectively the ciliopathies represent a large part of the genetic pathology. Numerous genes, over 200, have been linked in the last years with these diseases due to development, extend and optimize of the next generation sequencing technologies and due to impressive advance of experimental modeling, cell biology and proteomics. Other 250 genes are proposed as candidate for pathogenesis of ciliopathies.

The general part of this study is structured on two main capitols: the first capitol offers a general overview of the investigated field, presenting the recent knowledge about cilium and its relation with human pathologies, the structure and function of primary cilium, background and complex genetic mechanism underlining the ciliopathies, the huge clinical heterogeneity of this group of disorders with multiorgan involvement, clinical diagnosis algorithm and classification of ciliopathies. The first subchapter presents a short history and the course of cilium since its first identification, in 1674 by Dutch biologist Antoni van Leeuwenhoek. The researcher, while studied a rain water sample with a home-made rudimentary microscope brought the first evidences about protozoa and the cilia that they use for locomotion. His observation regarding "*animalicules"* it would revolutionize the scientific world beyond centuries. [1] During time, once the advances of microscopy technologies, various components of the little organelle have been detected. Furthermore, several genes whose haploinsufficiency were linked with various human pathologies, have been identified by studying the ciliary components. [2, 3] 2000 years mark "the golden age" for the study of primary cilium and its related disorders, shortly several diseases were associated with the little organelle such as: polycystic kidney disease, nephronophthisis, retinitis pigmentosa or Bardet Biedl syndrome. [4, 5]

The first evidences that linked primary cilium with human pathology were published in 2002, showing the ciliary localization of polycystin-2 and its implication in polycystic kidney disease. [6] Subsequently, an increasing interest and a plethora of studies demonstrate the complex role of primary cilia, redefining and remodeling this field.

The second subchapter compares the almost similar structure of primary and motile cilia. The structure of two types of cilia consists of a microtubule backbone, named axoneme, surrounded by a matrix and covered by ciliary membrane, which is continuous from the plasma membrane. At the base of this scaffold, a specialized centriole called basal body, helps anchor the cilium to the cell. [7] Further is highlighted the specialized ensemble involved in motility, which differentiates the two types of cilia and that is composed of a central pair of microtubules reinforced by radial bridges plus some accessory arms of nexin and dynein (9+2 type). [8] A special attention has been paid in this subchapter to the function of primary cilium, a function that was unappreciated for centuries, the cilium being considered "*a rudimentary organelle, with a transitory existence and an unknown function*". [9] There are presented the extend of cellularly processes across the cilium that situate the little organelle in the middle of the physiological events, which contribute to embryogenesis and cellular homeostasis after birth. [10] The first described is an ultra-specialized bidirectional movement of protein cargo which are continuously trafficked within ciliary compartment – intraflagelar transport [11], and next are highlighted each signaling pathway underpinning ciliary function: *Hedgehog (HH) signaling*, *Wingless (Wnt) signaling*, *Notch signaling*, *Receptor tyrosine kinases (RTK) signaling*, *Extracellular matrix (ECM) signaling*, *(TRP) signaling*, *The G protein-coupled receptors (GPCR) signaling*, *(TGFβ) signaling*, *The mammalian target of rapamycin (mTOR) signaling*, *Salvador-Warts-Hippo (SWH) signaling*, *Brain derived neurotrophic factor (BNDF) signaling*, and *polycystin*, *serotonin*, *somatostatin*, *vasopressin*, *melanin signaling*. [12-16]

In the next subchapter is approached the huge clinical heterogeneity and the complex multiorgan involvement, which are hallmarks for the ciliopathies, by presenting the histological, imagistic and clinical aspects starting with a core of features including ocular, renal, brain and skeletal findings. [17-22] This core of features is associated with signs and symptoms that could affect any organ or system (collected after an overview analysis of Online Mendelean Inheritance in Man database – OMIM: https://omim.org/). The severity of ciliopathies is variable ranging from a mild to a very sever, lethal phenotype. Moreover, organ involvement is also variable spanning a complex presentation from one organ affected, such as retinal dystrophies or nephronophthisis (NPHP) and polycystic kidney disease, to

multiorgan implication that could be fall into several categories, based on the extend of which an organ is involved: (Figure 1) [23]

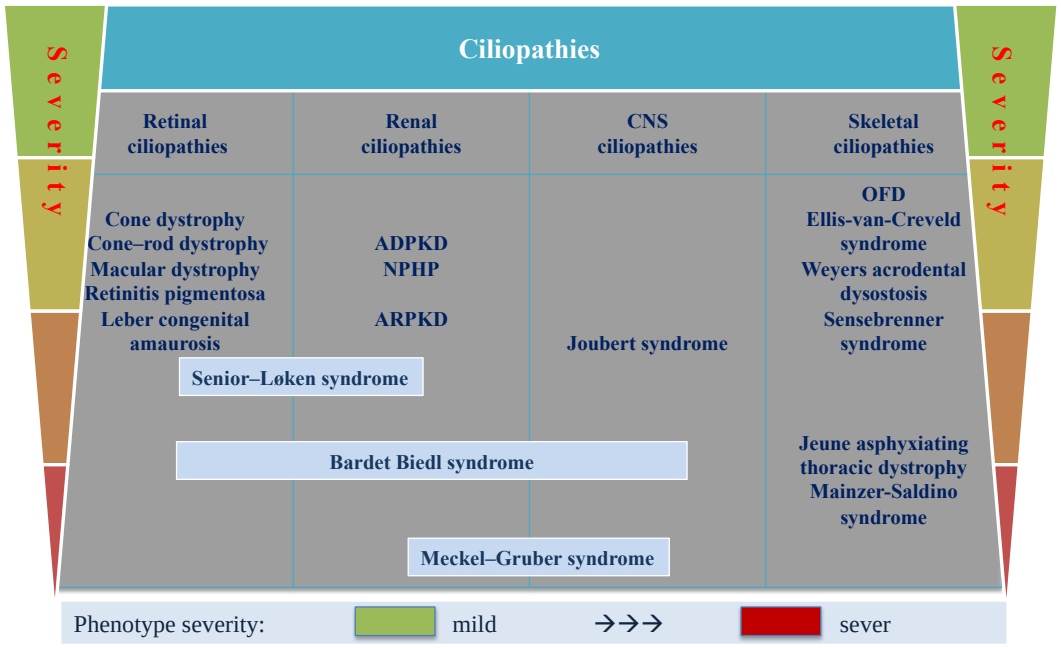

Figure 1: classification and severity of ciliopathies

Ocular ciliopathies (retinal) comprise: Alström Syndrome (AS) characterized by progressive retinal degeneration, sensori-neural hearing loss, obesity and diabetes mellitus and at the border with renal ciliopathies, Senior Loken Syndrome(SLS) defined by retinal dystrophy and nephronophthisis. [24] This category of disorders is caused by impairment of function or morphogenesis of some specialized sensory cilia located in retina that constitutes the outer segment of photoreceptors. Rhodopsin is one of the proteins that are shuttled along these specialized primary cilia by the IFT particle. Consequently, defects of different IFT proteins lead to the accumulation of rhodopsin in the outer segment of photoreceptors resulting in the impairment of their development or in the triggering of their apoptosis. All these processes are phenotypically reflected as retinal degeneration. [24, 25]

Renal ciliopathies include BBS and, transitioning to CNS-related ciliopathies, Meckel-Gruber (MKS). [26, 27] Primary cilia are lining the nephrons tubules and collecting ducts in the kidney and they are sensitive to the urine chemical, osmolar or flow changes. Thus, defects in several signaling pathways such as: GPCR signaling or mTOR signaling mediated by decreased or flow-related calcium concentration are the underpinning mechanisms for cyst formation. The cyst occurring is explained, also, by the unbalance

between canonical and non-canonical Wnt signaling that affect the polarity of renal epithelial cells. [28]

CNS-related ciliopathies include Joubert syndrome (JS) and Joubert-like syndromes classified as follows: 1) Classic JS characterized by hypotonia, developmental delay, abnormal eye movements, breathing abnormalities, ataxia and intellectual disability; 2) JS with ocular anomalies that could be either retinal dystrophy or Leber congenital amaurosis (LCA); 3) SJ with nephronophthisis; 4) SJ with oculorenal defects, also known as cerebello-oculorenal syndrome, that is characterized by SLSN (retinal dystrophy, LCA and NPHP) associated with molar tooth sign (MTS), and Dekaban-Arima syndrome (cerebro-oculo-hepato-renal syndrome) defined by chorioretinal coloboma or retinal dystrophy, PKD, MTS and hepatic fibrosis in some cases); 5) JS with congenital hepatic fibrosis; 6) hepatic fibrosis may also be associated with chorioretinal coloboma, constituting COACH syndrome; and 7) JS with orofaciodigital defects, and/or palate and polydactyly, also known as orofaciodigital syndrome type VI. [29, 30]

Cerebellar development is regulated mainly by *Wnt* signaling pathway, thus knockdown of *Wnt* molecules may be responsible for cerebellar vermis hypoplasia, a component part of the MTS. Likewise, other cilium-dependent pathways such as: *Sonic hedgehog* (*SHH), PDGFRα* or *GPCR*, are involved in proliferation, migration and differentiation of neurons, hence modulating the normal development of brain. Malfunction of any of these pathways lead to malformation during cortical development or midline defects. [31, 32]

Unlike the mechanism of brain defects occurrence in CNS-related ciliopathies, in MKS the mechanism underpinning the brain anomalies is different. While in MKS, characterized by loss of cilia and caudal neural tube dorsalization, was shown to be associated with down-regulated Wnt/b-catenin signalling, JS-like, associated with aberrant cilia structure and mild neural tube ventralization, was demonstrated to be associated with up-regulated Wnt/b-catenin signalling, and increased non-canonical Wnt and Shh signalling. *[33]*

Skeletal ciliopathies encompass two different subgroups:

The first with major skeletal involvement including craniofacial, thoracic cage and long bones, known as short-rib thoracic dysplasias with or without polydactyly (SRTD) or ciliary condrodisplasias which include *Ellis-van-Creveld syndrome (chondroectodermal dysplasia, EVC), Weyers acrodental dysostosis (WAD)* and *Sensebrenner syndrome (cranioectodermal dysplasia, CED), Jeune asphyxiating thoracic dystrophy (JATD)* and *Mainzer-Saldino syndrome (MZSDS), Saldino-Noonan syndrome (SNS), Majewski syndrome (MS), Beemer–Langer syndrome (BLS)* and the second subgroup, with milder involvement of skeletal

system, orofaciodigital syndromes. [34, 35] *IFT* and *HH* signaling are the major modulators of bone and cartilage development. Dysregulation of this processes in chondral primary cilia may affect either chondrocyte maturation or premature differentiation and decreased proliferation of chondrocytes during the ossification process. As a consequence of these disturbances, numerous skeletal anomalies may manifest, including polydactyly, shortening of the ribs or long bones, defects of long bone growth plates and craniofacial abnormalities. [36, 37]

The next subchapter is dedicated to the clinical diagnosis algorithm and to the molecular confirmation of ciliopathies. Given the various overlapping features and remarkable genetic heterogeneity as well as the complex genetic mechanism underpinning these disorders, establishing the diagnosis is not easy to accomplish. However, a clinical diagnosis algorithm was proposed by Beales et al. starting with the three main clinical signs: retinal dystrophy, polydactyly and kidney disease. These signs accompanied by skeletal abnormalities could easily drive the diagnosis to one of the ciliary skeletal dysplasias. If any of ectodermal defects are identified, the oral-facial-digital syndrome (OFDS) or cranioectodermal dysplasia should be suspect. In their absence, the diagnosis of short-rib polydactyly syndrome is suggested. Imagistic detection of MTS is a major clue for diagnosis of JS or JS-like, while the obesity should raise the suspicion of BBS or AS. [38]

The genetic substratum of ciliopathies is complex, over 200 of genes being associated with their pathogenesis, of which over 150 genes are related with primary ciliopathies. Furthermore, other over 250 genes are proposed as candidate for ciliary related pathology. [39] Beside classical mendelian inheritance of the ciliopathies, known to be in an autosomal recessive fashion [40], a growing number of evidences suggest the non-mendelean character of this disorders: the oligogenicity. [41, 42] In addition, several genetic mechanisms that contribute to the phenotype delineation and to the severity of symptomatology as well as to the marked variability within and between families have been highlighted: locus heterogeneity, *copy number variants* (*CNVs*), multiple allelism and genic epistasis . [43-47] Recent studies approached the less known basis for phenotype variability such as transposon-mediated mutagenesis, epigenetic modifier and aberrant splicing. [48-50] Moreover, it was emphasized the effects of the types of mutations (i.e. *non-sense, frameshift*) on expression (i.e. hypomorphic) and on their protein modification (quantitative, structural or functional). [51]

In the second chapter of the first part it was chosen for example one of the most dissected ciliopathies that is a model for the entire of its class: ***Bardet Biedl syndrome.***[52] It was

presented a short history of BBS, since first description 1866 by John Laurence and Robert Moon, to the reports beyond decades of the two doctors whose name defined the disease George Bardet and Arthur Biedl, the last one born on the Romanian territory, in Comloșul Mic near Timișoara. In 1925, Solis-Cohen and Weiss considered that the clinical pictures described in the three studies was similar and redefined the disorder as Laurence-Moon-Biedl syndrome. Move forward in 1980 the disease it was again revised and split into two current syndromes: Bardet-Biedl and Laurence-Moon. [53-55]

Being a rare disease with a prevalence of 1:160.000 in European population, next, it was analyzed the frequency of BBS in other populations. [56-58] In the following subchapter it was emphasized the main clinical findings which are primary criteria for clinical diagnosis: retinal dystrophy, postaxial polydactyly, central obesity, urogenital anomalies, learning difficulties and kidney disease. [59-61] Additional clinical findings: neurodevelopmental and behavioral abnormalities, liver involvement, endocrine and metabolic abnormalities, oral-dental anomalies, craniofacial dysmorphic features, cardiovascular impairment, and hearing loss, constitute the secondary criteria for clinical diagnosis.[62-64] Moreover it was highlighted the clinical diagnosis algorithm by the presence of four major (primary) findings or by combination of tree major features with two minor (secondary) symptoms. [65]

Next, were described the 26 de genes linked, so far, with SBB pathogenesis, respectively *BBS1, BBS2, ARL6, BBS4, BBS5, MKKS, BBS7, TTC8, PTHB1, BBS10, TRIM32, BBS12, MKS1, CEP290, WDPCP, SDCCAG8, LZTFL1, BBIP1, ITF27, IFT74, CFAP418, NPHP1, IFT172, SCAPER, SCLT1* and *CEP164*, being analyzed their chromosomal position (Figure 2), the location and the interaction of their protein products within ciliary compartment (Figure 3), their ciliary function [66-75] as well as the number of pathogenic variants responsible for BBS reported so far in „The Human Gene Mutation Database": http://www.hgmd.cf.ac.uk/ac/gene.php, the types of this variants and the other phenotypes related to BBS genes.

The following chapter approach the biggest challenge in studying ciliopathies: the genotype-phenotype correlation. There are some reports that established some correlation for the genes that are most frequent involved in BBS pathogenesis such as: *BBS1, BBS2, BBS4, BBS8, BBS6, BBS10, BBS12* although these evidences are able to explain only in part the huge variability due to the complex genetic mechanism and the limited number of patients bearing the exact same genetic modification.

Moreover, the difficulties of a long term follow up or the clinical heterogeneity mirrored in

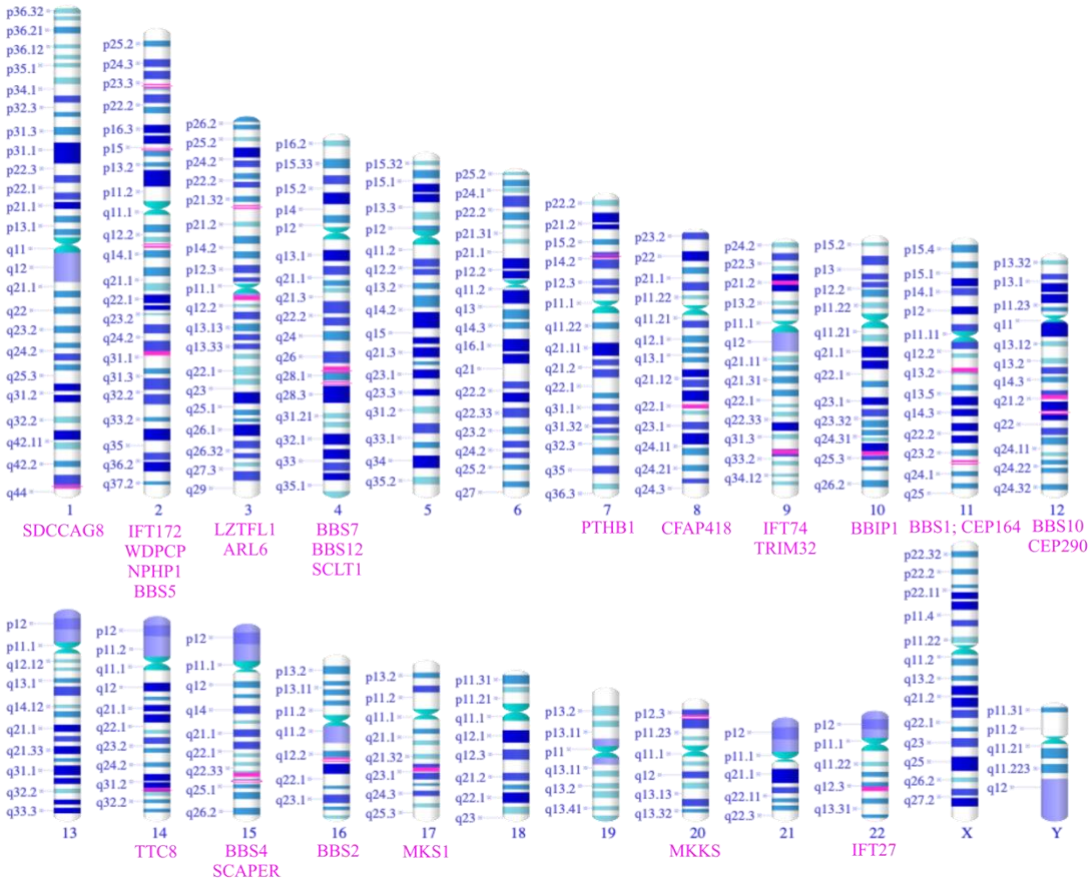

Figure 2: Ideogram of chromosomes with location of BBS genes (marked in pink) _drawn with ***Genome Decoration Page*** _ https://www.ncbi.nlm.nih.gov/genome/tools/gdp

the different age of occurring the symptoms may complicate the investigation of clinical presentation related to genetic substratum. [76-80]

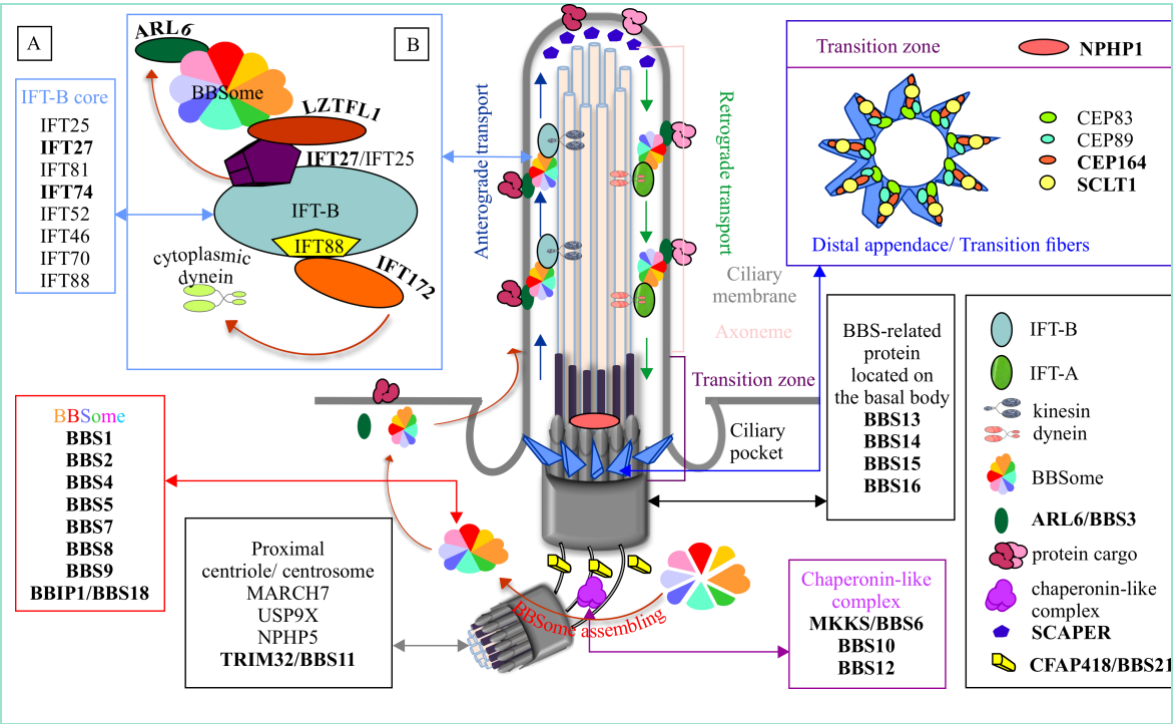

Figure 3 Location and interaction of BBS proteins within cilium_BBS protein are in bold

The overlapping clinical presentation with BBS is characteristic to McKusick-Kaufman, Alström, Cohen, Simpson-Golabi-Behmel, Prader-Willi or Laurence Moon syndromes and are the subject of differential diagnosis that is detailed in the following subchapter. [81-86]

Management of BBS, described next, it should be done by a multidisciplinary team and it should be focused by amelioration of symptoms and by anticipation and periodic screening of comorbidities. Thus, establishing a regular program of physical exercises combined with restricted low-calorie diet is required to control weight gain; early enrollment in an educational training for the blinds, even before the complete vision loss, is essential for integration into society and for raise the life quality; treatment of renal dysfunction in early stages with an adequate diet and by limiting comorbidities such as hypertension, diabetes or metabolic syndrome may slow the progression of the disease, whereas in the advance stages the dialysis and kidney transplantation should be considered. [87]

In addition to this measures that are intended to improve the lifestyle and life quality of BBS individuals, in this subchapter was highlighted the research advances, the clinical trials using various molecules, which target the ciliary signaling pathways and their receptors that are ongoing such as: inhibitors of melanocortin to control obesity or vasopressin R2 receptor antagonists, mTOR inhibitors and multi-kinase inhibitors to improve the kidney disease. [88] Personalized medicine for treatment the BBS patients is still in the early stages, however, research on gene replacing and gene editing therapy, aberrant splice-correcting and

exon-skipping therapy, read-through therapy that target the correction of non-sense variants, have been successful in vivo experiment and open new perspective in the future therapeutical approach of BBS patients. [89-92]

In the last subchapter of the general part was approached the genetic counselling and it was highlighted the risk of inheritance as well as the opportunities of prenatal diagnosis. [64, 93]

RESEARCH

Aim

The main objective of this research was to study a Romanian cohort of BBS patient. To achieve this purpose, it was identified those patients who fulfilled the clinical consensus criteria, it was analyzed the personal and familial history, the familial pedigree, and the clinical pictures. Next step it was to collect the biological sample, to identify the genetic substratum of disorder and to compare the results with previous research.

Methodology

The patients were recruited from the majority of the University Centers in the country with help of our collaborators from the Genetics, Pediatrics, Pediatric Nephrology, Pediatric Neurology Departments. The patients and their legal guardians were informed about the methodology and it was obtained their accord for participation in this research by signing the written informed consent.

In the study were included those patients who fulfilled the clinical diagnosis algorithm for BBS established by Beales et al, 1999 (Table 1) [65]. There were no exclusion criteria.

Biological samples, meaning EDTA-treated peripheral venous blood, were used for genomic DNA extraction using the dedicated kits according with the manufacturer's protocols. Genetic tests for identification of common mutation following by next generation sequencing were performed by the Center for Human Disease Modeling, Duke University, Durham, United States of America, and by the Advanced Center for Translational and Genetic Medicine, Ann & Robert H. Lurie Children's Hospital of Chicago, United States of America. All the results were confirmed by Sanger sequencing.

Table No.1: Consensus criteria for clinical diagnosis of BBS [65]

Primary criteria	*Secondary criteria*	*Diagnostic*
Retinian dystrophy Polydactyly Obesity Genitourinary anomalies Renal anomalies Learning dificulties	Developmental delay Speech delay Ataxia/ poor coordonation Behavioral abnormalities Oral/dental abnormalities Craniofacial dysmorphism Metabolic syndrome Endocrine abnormalities Brachydactyly/syndactyly Cardiovascular abnormalities Liver disease Gastrointestial diseases	*4 Primary Features or 3 primary and 2 secondary features*

Case presentation

25 patients, 9 males and 16 females were selected, of which 2 are siblings. Another case comes from a family with two affected siblings but younger sister was deceased at the time of enrollment. 4 of the cases come from the consanguineous families. The age at the time of enrolment varied between 2 months and 43 years. 76% of patients were under 18 years old. Most patients had the age between 1 and 10 years, 76% respectively. 8% were under 1 year old, 8% were between 30 and 40 years old and 4% were over 40 years.

The age of the clinical diagnosis was, in the majority of cases, in the first year of life, which is lower comparing with other studies. Previous research indicated that the age at diagnosis varied between 5 and 10 years, age at which rod cone dystrophy became symptomatic. [65, 94] However, suggestion that BBS could be suspicioned even antenatally or immediately after birth were based on the presence of polydactyly, renal or genitourinary malformation. [95, 96] According with these reports, the suspicion of BBS diagnosis was raised antenatally in one of our cases, while in two cases the diagnosis was made shortly after birth. Other two cases were diagnosed later at 11, and 12 years, respectively.

Major clinical findings were distributed in our cohort as follows (Figure 4):

Polydactyly were observed in 23 of the cases (92%). The accessory digits were present in all four limbs in 13 patients (52%), only on the feet in 4 patients (16%) or only on the hands in 1 patient (4%). In 1 case (4%) the polydactyly was present in both hands and one

foot, in other 1 patient (4%), extra digits were present in one hand and both feet, while 3 cases (12%) exhibited polydactyly only in the left limbs. The higher percentage of polydactyly in our cases, comparing with previous reports (63-81%) [64, 94, 97] may be explained by a limited number of patients enrolled in the study, however could also suggest a lower rate of diagnosis in BBS patients without polydactyly.

Obesity was recorded in 21 of our patients (84%), in accordance with literature (72-92%) [65, 94, 97]. Typically, the birth weight is normal and the weight gain commences in the first year of life. [94] In our cohort, the birth weight was towards to the lower percentiles in 10 patients (40% of the cases), however, within the normal range. Of these, 3 children (12%) were on percentile 1. 7 children (28%) had a normal birth weight. 3 children (12%) had birth weight towards to the upper percentile, while 4 children (16%) had birth weight above the upper percentile.

Retinal dystrophy was present in a lower percentage in our cohort, 64%, meaning 16 cases, comparing with previous reports (93-94%) [64, 65, 97]. This inconsistency may be due to the young age of some patients in our study, or due to the small number of cases enrolled. Remarkable, the visual deficit was observed in the first year of life in two patients and in the first three years of age in other two cases, earlier than previous reports.

Genitourinary anomalies were noted in 84% of our patients (21 cases), consistent with literature (59-98%) [64, 94, 97]. All males had small penile size, while 31% (5 cases) of the females had vaginal atresia and other 37% (6 cases) had hypoplastic genitalia.

Renal involvement was identified in 11 cases (44% of our patients), in accordance with previous reports (24-53%). [64, 65, 97] The most frequent anomaly was hydronephrosis seen in 5 patients (20% of cases), followed by polycystic kidney in 4 cases (16%). Other structural anomalies, i.e., hypoplastic or atrophic kidney, were detected in 3 patients (12% of cases). Renal dysfunction affected 7 patients (28% of cases) of which e patients (12%) it has progressed to end stage renal disease, and 1 patient (4%) undergone renal transplantation. Learning difficulties were present in a higher percentage in our patients 80% comparing with previous studies (61-66%) [64, 97], which may be due to the small number of patients enrolled, or due to different scale used in evaluation of the patients in our cohort.

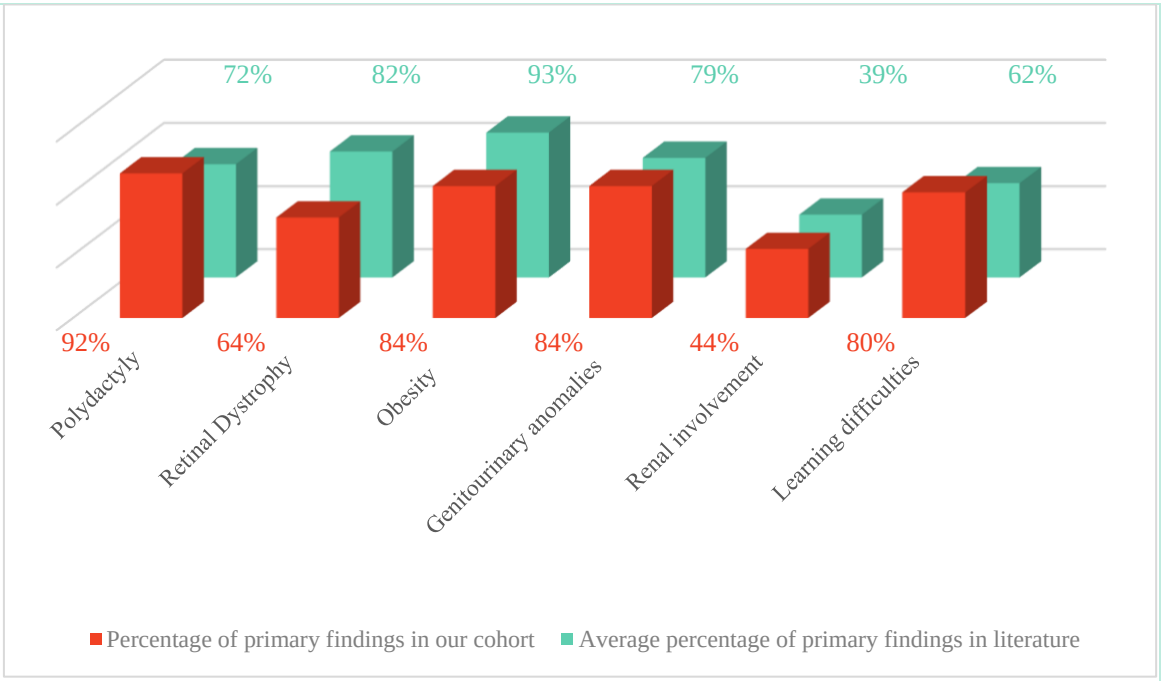

Figure 4: Distribution of primary clinical findings in our cohort comparing with literature

Main secondary features were present in our patients as following:

Psychomotor delay was noted in 18 cases (72% of our patients), language delay was observed in 16 patients (64%), while psychiatric conditions, consisting in behavioral abnormalities, autistic spectrum disorder, hyperkinesia, affected 7 cases (28% of patients). Cardiovascular defects were present in 7 patients (28%). These features are consistent with previous reports, including psychomotor delay (50-81%) [64, 65], language delay (54-81%) [65, 97], cardiovascular involvement (7-29%) [64, 97], while psychiatric condition are slightly below previous reports. [64, 65, 94] (Figure 5)

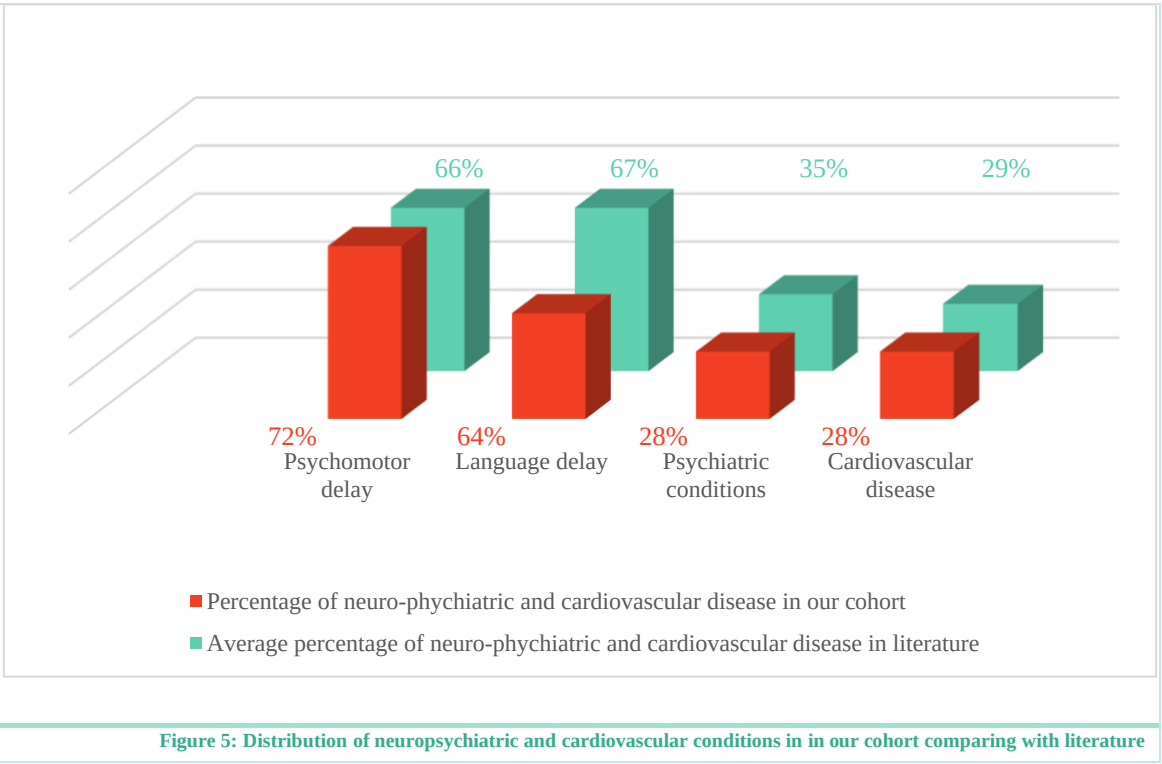

Figure 5: Distribution of neuropsychiatric and cardiovascular conditions in in our cohort comparing with literature

Metabolic syndrome was detected in 8 patients (32%), while type 2 diabetes and hypothyroidism were recorded in 6 cases (24%), and 7cases (28%), respectively. Hepatic disease affected 9 patients (36%). Below average was metabolic syndrome (54%) [64], while hypothyroidism was much frequent in our patients comparing with literature (20%) [64]. These findings could be assigned to the small number of patients enrolled in the research; however, it may also suggest a hallmark of our population modulated by diet or by environmental influence. (Figure 6)

Other ocular and digits anomalies, besides those that constituted the primary findings, were also seen in studied patients. Digits anomalies including brachydactyly was observed in 11 cases (44%), syndactyly was remarked in 6 cases (24%), of which one patient presented bone syndactyly, conic fingers were seen in 6 patients (24%) and in 1 case (4%) presented an unusual anomaly for BBS patients, bilateral hypoplasia of second phalange of 5^{th} fingers.

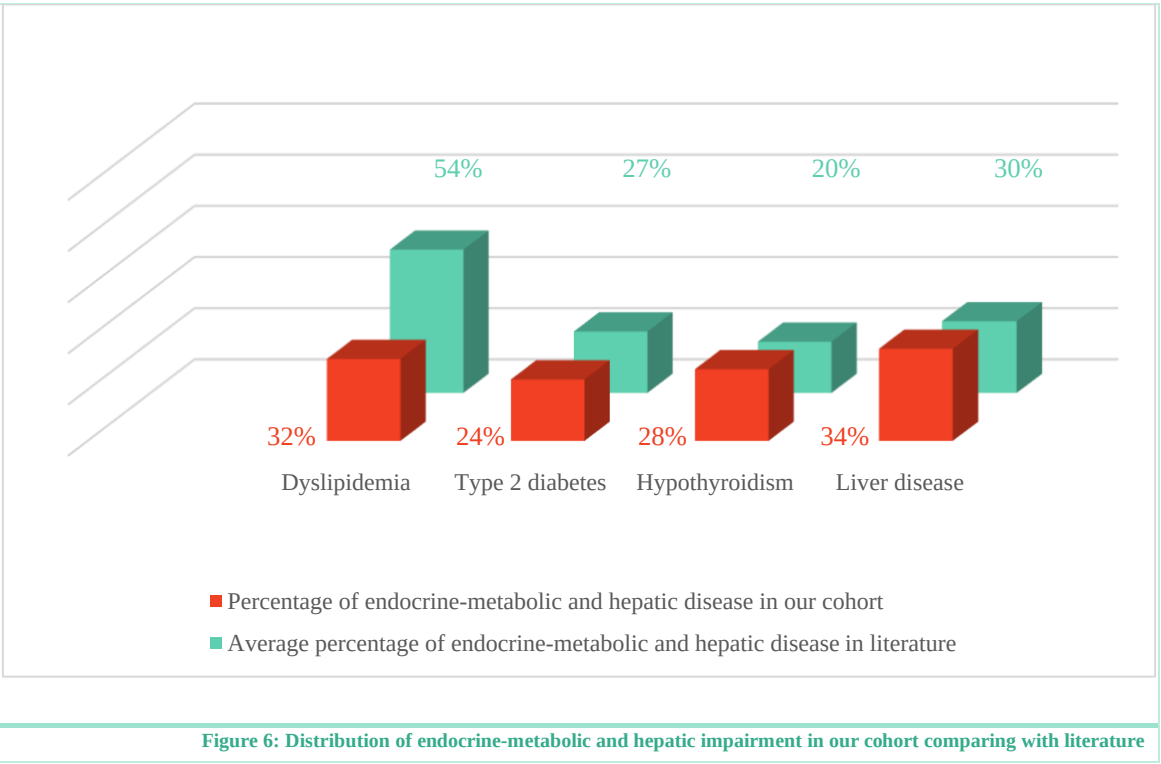

Figure 6: Distribution of endocrine-metabolic and hepatic impairment in our cohort comparing with literature

Ocular anomalies include myopia seen in 5 patients (20%), strabismus detected in 5 cases (20%), nystagmus identified in 5 patients (20%) whereas astigmatism was present in 5 patients (20%). Cataract, optic atrophy was remarked in a lower percentage 4%, one patient each, respectively.

Results and discussions:

A pathogenic variant in known BBS genes was identified in 20 cases (80%), respectively in *BBS1*, *BBS2*, *BBS4*, *BBS5*, *BBS7*, *BBS10*, *BBS12*. Of these, 1 case was heterozygous, which was not sufficient for confirmation of clinical diagnosis. In 5 cases (20%) haven't been identified any pathogenic, likely pathogenic or with uncertain significance variants in BBS genes. (Figure 7) The identification percentage of the genomic cause in our patients are consistent with literature. [52, 97, 98] Surprising, only one common heterozygous variant in *BBS1* has been identified. Furthermore, in BBS1 has been also detected a variant less reported in BBS patients, an intragenic deletion (CNV – copy number variant). Therefore, *BBS1* was found mutated in 8% of our patients, in disagreement with previous studies that reported much higher percentage of mutation in BBS1. (51% in Europeans, or 23% in Caucasians) [52, 61]

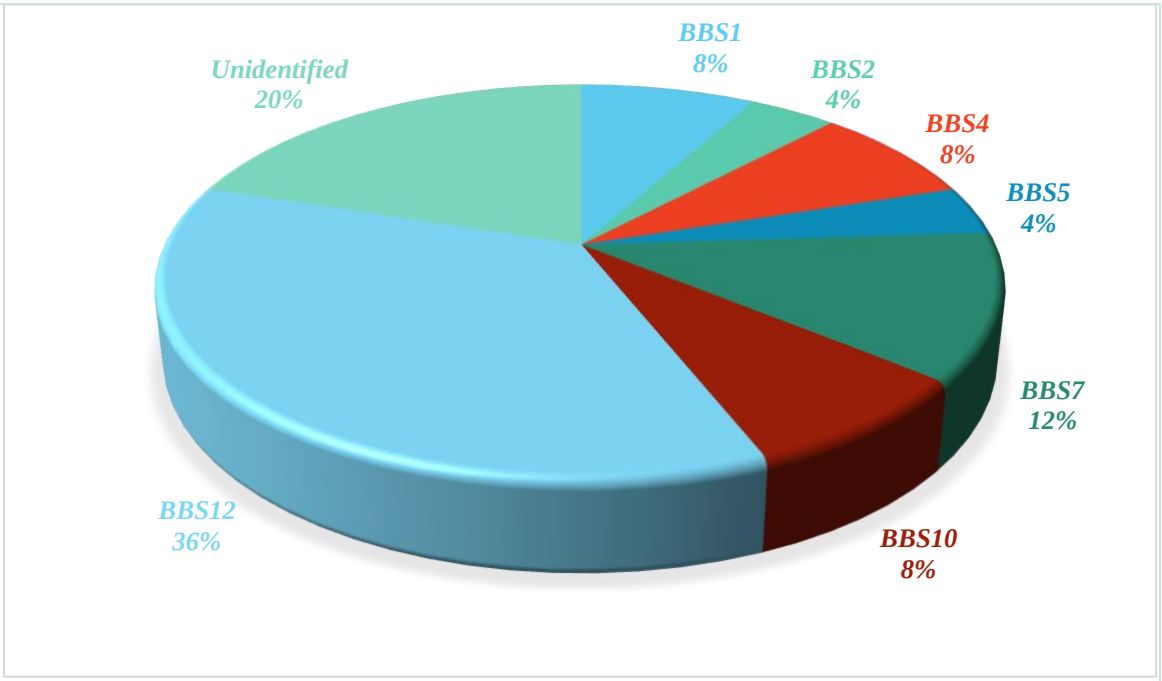

Figure 7: causative BBS genes in our cohort

The common variant of Europeans in *BBS10* was not identified in our cohort, however other two variants were detected. Thus, the second gene as a frequency (20-33%) [52, 61] responsible for BBS was found mutated in only 8% in our patients. Moreover, another striking finding in our cohort was the identification of a homozygous pathogenic variant in *BBS12* in 8 patients, meaning 32% of the cases. This variant has a low-frequency and has been reported previous only in 4 patients, one of them being also Romanian and increasing the number of Romanian patients who are harboring this variant to 9 cases. The other cases are one Italian and two French. [79, 99, 100] This findings suggest, certainly, the existence of a founder mutation in Romanian population. Furthermore, detection of this genomic change in all Romani patients enrolled in this study shows that it is specific and common in Romani population from our territory. In addition, pathogenic variant identified in *BBS7* may be also a consequence of a founder mutation, its frequency being much higher in our population (12%) comparing with literature (1,5-3%). [52, 61, 98]

Out of the 20 patients in whom pathogenic variants were identified, 14 were homozygous (74%), 4 cases were compound heterozygous (21%) whereas 1 patient was heterozygous (5%).

The types of genomic changes identified in our cohort were heterogenous and included: 8 null variants (*non-sense*), meaning 34%, 7 (28%) *missense* variants, 5 (21%)

frameshift variants, o 1 *splice site* variant, 1 intronic variant and intragenic (*CNV*). (Figure 8)

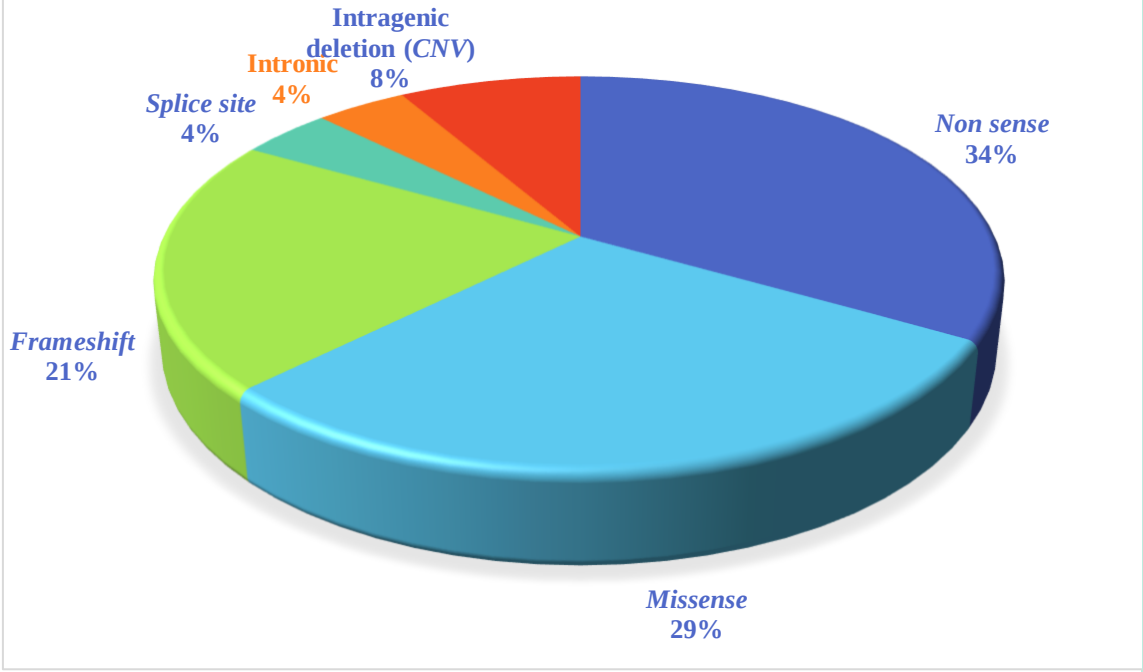

Figure 8: Types of pathogenic variants identified in our cohort

Our study confirms the high clinical heterogeneity of BBS, inclusively intrafamilial, by exemplifying it in patients that bearing the identical variant in BBS12, respectively Arg355*, of which two are siblings (cases 10 and 11 – Table 2). Hence, 4 patients presented postaxial polydactyly in all four limbs, 1 patient only in the feet, 1 only in the left limbs, 1 only in the left foot while in 1 patient extra digits were present in right hand and both feet.

Out of variants identified, this variant (Arg355*) is associated with the highest values of weight. The younger patient in this group, a 2-month-old girl, had the birth weight above the 99th percentile, whereas the higher weight in the whole cohort was also that of a patient who has this mutation, respectively 160 kg. The weight in rest of the group with Arg355* variant had also significant exceeds of standards from 3, 2 to 13,95 deviations.

Table no. 2: Summary of major clinical findings Arg355 cases*

Case No.	*Age*	*Polydactyly*	*Obesity*	*Retinal dystrophy*	*Hypogonadism / Genitourinary Anomalies*	*Kidney disease*	*Cognitive difficulties*
6	6	H, F	+7SD	Yes	Vaginal atrophy, hEG	No	sever
10	21	H, F	+3,2SD	Yes	hP	Chronic nephropathy	
11	16	F	+19SD	Yes	No	No	mild
17	7	H, F	+4,3SD	Yes	hP	Left Hydronephroses	
18	9	LH LF	+6,3SD	Yes	hEG		sever
20	2 months	LF	>pc99	No	Vaginal atresia	Bilateral hydronephroses Renal dysfunction	moderate
21	13	RH, F	+13,95SD	Poor vision	hEG	Interstitial nephrite, fibrosis, glomerulosclerosis	moderate
22		H, F	+11,63SD	Poor VISION	hP	No	moderate
PRC	5 months	F	>pc95	NO	hP	No	No

PRC: previously reported case [99], F: feet, H: hands, hEG: hypoplasia of external genitalia, hP: hypoplasia of penis , LH: left hand, LF: left food, pc: percentile, SD: standard deviations

Retinal dystrophy was confirmed in 5 patients of the group with Arg355* variant, while 2 patients presented visual deficit but haven't been tested. The younger patient cannot be investigated yet due to her age.

All patients presented urogenital anomalies, while renal disease was various and it was seen in 5 patients. In the 6th patient the renal disease just started with slightly increased levels of urea and creatinine

Cognitive deficit varied as severity, in 1 case being mild, in 3 cases being sever and in 1 patient being moderate. Development delay was recorded in all patients, however was various as severity or as an involvement of different components of development. Thus, 2 cases presented severe language impairment, showing (echolalia, bradylalia, dyslexia, and a limited vocabulary) whereas case number 18 says one word at the age of 9 years. In other 2 cases the delay was more prominent either on the motor area, case 10, or on the language, case 17. In 2 case various behavioral abnormalities were noted.

Cardiac malformations were noted in one of our cases and were also reported in previous published case. Interestingly, these two cases were the only who presented politely. The two siblings had hypertension, type II diabetes, liver steatosis with high level of hepatic enzymes. They presented also dyslipidemia, being mixt in sister and only high level of triglycerides in brother. Hypertriglyceridemia was recorded in one more case (case 17). Various ocular anomalies were remarked in the 2 patients who had the most severe general presentation including nystagmus and cataract. (Table 3)

Table no. 3 Summary of minor clinical findings Arg355 cases*

Case No.	*GDD*	*Psychiatric conditions*	*Cardiovascular disease*	*Dyslipidemia*	*Diabetes*	*Hypothyroidism*	*Miscellaneous*
6	Yes LD	BA	No	Yes	No	No	
10	Yes Most motor	No	HTA	HT	Yes	No	Steatosis HE↑
11	Yes	BA	PVM HCMP HTA	Mixt	Yes	Yes	Nystagmus Politely Steatosis HE↑
17	Yes Most language	No	No	HT	No	No	
18	LD	No	No	No	No	No	Cataract
20	No	No	No	No	No	No	
21	Yes	No	No	No	No	No	
22	Yes			No	No	No	
CPA	hypotonia	No	HSVS				Politely

HCMP: hypertrophic cardiomyopathies, PRC: previous reported case [99], HE: hepatic enzyme, SLVH: hypertrophy septal and left ventricular hypertrophy, HT: hypertriglyceridemia, HTA: hypertension, GDD: global developmental delay, LD: language delay, BA: behavior anomalies

In our cohort, inheritance was in an autosomal recessive fashion. All parents available for testing were found heterozygous for the variants identified in their descendants. Several siblings of the probands were also tested. No triallelic or modifier variants have been

identified. Thus, the phenotype variability may be explained by the genetic background of each individual or by epistasis interactions and epigenetic modifiers. [89]

The healthy siblings had a 50% risk to inherit the mutant allele. Therefore, some of they being found heterozygous. Their risk to have an affected child depends on the genetic background of the partners or by the contribution of *de novo* variants in BBS genes. Thus, genetic testing of spouse or prenatal diagnosis, consisting in amniocentesis and next generation sequencing of fetal DNA, using a gene panel with all known BBS genes, in case of high biochemical risk associated with abnormal ultrasound signs, is strongly recommended.

Conclusions

This study is the first analysis on a cohort of Romanian Bardet Biedl syndrome patients, who were recruited from the majority of the University Centers in the country.

The limitations of the study are the absence of a national database of rare disease, the decreased incidence of BBS that explain the small number of patients enrolled, the difficulty of performing molecular testing due to the complex genetic mechanism that underpinning BBS and consequently of increased costs.

In the study were enrolled 25 patients with ages varies between 2 month and 43 years and phenotypic evaluation of each patient confirms the intrafamilial variability and the extreme pleiotropy that is characteristic to BBS and to the entire group of ciliopathies.

Major criteria of clinical diagnosis were distributed in our cohort as following: polydactyly 92%, obesity 84%, retinitis pigmentosa 64%, hypogonadism and genitourinary malformations 84%, kidney disease 44%, cognitive deficit 80%. Some of this percentages are in agreement with the literatures including obesity, hypogonadism, and kidney disease whereas the others are either higher in our study such as polydactyly and cognitive impairment or lower as retinitis pigmentosa.

Genetic investigations were identified a pathogenic variant in 7 of BBS genes, respectively: *BBS1, BBS2, BBS4, BBS5, BBS7, BBS10, BBS12,* in 80% of the patients. Variants identified were 14 homozygous (74%), 4 compound heterozygous (21%) whereas 1 patient was heterozygous (5%) insufficient for the molecular confirmation of the BBS.

Surprisingly, in our cohort was not identified common variants for Caucasian or European, respectively Met309Arg in *BBS1* or C91fsX95 in *BBS10*, with one exception that of single heterozygous variant (in *BBS1*).

Unexpectedly, a recurrent variant in *BBS12*, Arg355* was identified in 8 of our patients. The variant was reported before in only 4 cases, however it seems to be prevalent in our population (34%). Al Romani cases in our study harboring this variant. These findings suggest, certainly, the existence of a founder mutation in Romanian population

Of all pathogenic variants identified in our cohort 60% have been reported before in BBS patients or have been cited in populational databases, whereas the rest of 40% are new. This research strengthens the previous observations regarding the vast heterogeneity between and whiten families of BBS and of ciliopathies, in general, by point out the variability of severity or clinical presentation in those patients that bearing the same variant, including the two siblings.

Inheritance was in an autosomal recessive fashion, in our cohort. No triallelic or modifier variants have been identified. All parents available for testing were found heterozygous for the variants identified in their descendants, thus their risk to have another child affected with BBS is 25%.

The siblings that were found heterozygous have also a certain risk to born affected children. Their risk depends on the genetic background of the partners or by the contribution of *de novo* variants in BBS genes. Therefore, genetic testing of spouse and of fetuses is recommended.

Detecting the genetic substratum of the disease is of a great importance for management of these patients. Thus, periodic follow up of patients, especially of those whose age is below the threshold of occurring of the entire symptomatology, is crucial for anticipation and early identification of other organ involvement and may help in limiting the comorbidities and in improving the life quality of BBS patients.

Final thoughts:

Study of a rare disease is not easy, especially in the socio-economic context in Romania. Choosing this topic for my research it was a huge challenge, but passion for rare disease helped me to overcome any obstacle. Completing this study brings me not only personal satisfaction but also valuable data that would give me a different perspective on genetic diseases.

The knowledge gained, as a culmination of these years of study, will help me to take into account other considerations in approaching genetic pathology. I wouldn't be surprised if in the coming years, the complex genetic mechanism that characterizes ciliopathies would be extrapolated and would be associated with other genetic disorders.

Further directions:

1. The research will continue with the functional studies of a case who was molecularly confirmed as harboring a rare combination, which was not reported previously, an intronic variant and an intragenic deletion. At this time are ongoing the ARN and cell lines studies. Preliminary reports confirm the pathogeny of those genomic changes.
2. Further genetic investigation for molecularly undiagnosed patients with:
    a. Performing of microarray-based comparative genomic hybridization for detection of the large CNVs
    b. WES for parents and trio analysis aiming to identify possible candidate genes or other *de novo* genetic mechanism;
    c. Transcriptomics;
3. Proteomics of the new identified variants or of those reported previously but with no functional studies;
4. Haplotype analysis of the common BBS*12*, Arg355* variant.

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List of publication:

1. Clinical and genetic heterogeneity of primary ciliopathies (Review)

Focșa, I.O., Budișteanu, M., & Bălgrădean, M. (2021). International Journal of Molecular Medicine, 48, 176. https://doi.org/10.3892/ijmm.2021.5009

2. A case of Bardet Biedl syndrome caused by a recurrent variant in *BBS12*

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3. Clinical aspects of a rare disease: Bardet Biedl syndrome

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Lista cu papers and communication presented at national and international conferences:

1. Clinical and molecular characterization of a rare case diagnosed with Bardet Biedl Syndrome

I. O. Focsa, M. Budisteanu, C. Burloiu, L. C. Bohiltea, M. Balgradean; 53rd European Society of Human Genetics (ESHG) Virtual Conference, June 6-9, 2020; Publicat în : *Eur J Hum Genet* **28,** 798–1016 (2020). https://doi.org/10.1038/s41431-020-00741-5; *ISSN 1476-5438*

2. From cilia to complex disorders: The ciliopathies

I. O. Focsa, L. C. Bohiltea, M. Balgradean, *Annual Scientific Meeting of Victor Babeș Institute;* The 11th National Pathology Symposium, November 22 - 24, 2018, Bucharest, Romania, publicat în Abstract book, nov 2018, ISSN 2601-0771; ISSN-L 2601-07 www.totalpublishing.ro

3. The past, the present and the future in ciliopathies

Ina Ofelia Focsa, The 5th National Congress of Medical Genetics, September 26 - 28, 2018, Gura Humorului, Romania, publicat în Romanian Jurnal of Rare Disease, supl. 1/2018, pg 49, ISSN 2068-5882, www.rjrd.ro