Understanding the phenotype and preparing for therapeutics in Bardet-Biedl Syndrome

Submitted in partial fulfilment of the requirements of the Degree of Doctor of Philosophy

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Statement of originality

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Publications arising from this thesis/ research manuscripts

Mujahid S, Hunt KF, Cheah YS, Forsythe E, Hazlehurst JM, Sparks K, Mohammed S, Tomlinson JW, Amiel SA, Carroll PV, Beales PL, Huda MSB, McGowan BM. The Endocrine and Metabolic Characteristics of a Large Bardet-Biedl Syndrome Clinic Population. J Clin Endocrinol Metab. 2018 May 1;103(5):1834-1841.

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Abstract

Bardet-Biedl syndrome (BBS) is a rare pleiotropic ciliopathy characterised by rod-cone dystrophy, renal dysplasia, obesity, polydactyly, intellectual disability and hypogenitalism. This thesis explores the phenotype, natural history and therapeutic potential of BBS through the priorities of those who understand it best: the patients and carers.

The UK national BBS clinics provide the largest reported number of patients with BBS worldwide. I utilised this cohort to explore kidney disease, visual decline, obesity, morbidity and mortality in BBS as well as potential for testing pharmacological and lifestyle interventions.

I discovered that although renal dysplasia is common in this patient population, end stage renal failure occurs infrequently, usually in early childhood, and is almost unheard of in patients with the common *BBS1* p.Met390Arg mutation. Visual decline is genotype and mutation type dependent and patients with *BBS1* p.Met390Arg typically develop visual deterioration at a later age than other patients and progress more slowly. Although most patients with BBS experience severe obesity and elevated cardiometabolic risk, those carrying *BBS1* p.Met390Arg mutations remain at lower risk. The number of attendances at the paediatric national BBS clinics correlate positively with reduction in standardised body mass index (BMI-SDS).

In keeping with the hypomorphic p.Met390Arg clinical phenotype, exploration of the cellular phenotype revealed that fibroblasts from patients with *BBS1* mutations had a hypomorphic proliferation phenotype similar to control cells.

As a stepping stone to therapies, I demonstrate the effect of an exercise intervention on cognition and hippocampal neuroplasticity in BBS. Despite starting with a genetically

pre-determined hippocampal volume deficit, it is possible to generate a hippocampal volume increase through physical activity.

My findings serve to highlight the clinical and cellular hypomorphic phenotype associated with the common *BBS1* p.Met390Arg mutation, the natural history of BBS as well as the potential effects of lifestyle intervention. Although our understanding of this highly complex condition remains imperfect, I hope this work will help move BBS to the forefront for rare disease therapies.

Impact statement

Understanding the variability and natural history of rare diseases remains a conundrum for clinical geneticists, patients and scientists alike. It prevents us from providing accurate prognostic indicators and limits our ability to deliver both personalised medical care and robust outcome measures for therapeutic intervention. The work produced in this thesis aimed to address these challenges for people living with Bardet-Biedl syndrome (BBS).

This thesis demonstrates the clear hypomorphic effect of the *BBS1* p.Met390Arg mutation. This is particularly pertinent from the point of view of understanding the renal disease. We now understand that end-stage renal disease is much less common in BBS than previously thought, and particularly unlikely in patients with *BBS1* p.Met390Arg mutations. This has implications for service provision, whereby adults with *BBS1* p.Met390Arg mutations and no evidence of renal disease may not require regular nephrology input.

From the ophthalmological perspective this work delivers evidence for a genotype-dependent difference in age at onset and timeframe for visual deterioration. Crucially, there is a window of opportunity for therapeutic intervention, particularly for those patients with *BBS1* p.Met390Arg mutations. This is vital information for future trials in gene therapy and other forms of personalised eye therapy.

It is clear that obesity and the metabolic syndrome are significant contributors to morbidity and mortality in BBS. This work demonstrates the effect of the hypomorphic *BBS1* p.Met390Arg mutation as well as the successes of the expert obesity intervention offered by the national BBS clinics. The impact of this intervention could feasibly be applied to other obesity syndromes. Furthermore, the results achieved here have

provided a springboard for collaborative work with industry partners assessing the effect of new therapies for obesity and metabolic syndrome.

In the search for a cellular phenotype this thesis delivers support for previously published mouse model data demonstrating that cells deficient of functional BBS protein proliferate faster than control cells. Building on the evidence collected in this thesis has led to national and international collaborations aimed at investigating repurposed drugs and developing novel therapies.

The exercise trial showed that patients who participate in a physical activity intervention demonstrate a significant improvement in hippocampal volume in comparison to a control group. This exemplifies a non-invasive intervention which could be applied to other genetic disorders associated with hippocampal volume deficit.

My aspiration is that the impact of the work done in this thesis will continue to evolve. Two of the chapters addressed in this thesis have been published in high impact peer-reviewed journals and other manuscripts are in preparation. Furthermore, I have disseminated my work through publication of lay audience talks on the BBS UK YouTube channel, winning the 3 Minute Thesis for UCL and multiple cross-disciplinary teaching sessions.

The aim of this thesis was to have an impact beyond academia; improving clinical service provisions and quality of life for patients. Hopefully, the future will deliver the ultimate impact for people living with this condition: the development of novel, effective therapies.

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List of abbreviations

α-MSH	alpha melanocyte-stimulating hormone
AC3	Adenylate Cyclase-3
AD	Axial Diffusivity
AgRP	Agouti –Related Peptide
AMPK	AMP-dependent protein kinase
APC	Adenomatous Polyposis Coli
ARC	Arcuate nucleus
ASD	Autistic Spectrum Disorder
ASL	Arterial Spin Labelling
AXN	Axin
BBS	Bardet-Biedl syndrome
BET	Brain Extraction Tool
BDNF	Brain Derived Neurotrophic Factor
BMI	Body Mass Index
BMI-SDS	Body Mass Index Standard Deviation Score
BMP	Bone-Marrow-Protein
cAMP	cyclic Adenosine MonoPhosphate
CART	Cocaine and Amphetamine Regulated Transcript
CBF	Cerebral blood flow
CK1	Casein Kinase 1
CKD	Chronic Kidney Disease
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CNF	Ciliary Neurotrophic Factor
CRP	C-Reactive Protein
CSF	Cerebro-Spinal Fluid
DDD	Deciphering Developmental Delay study
DEXA	Dual Energy X-ray Absorptiometry
DISC1	Disrupted-In-Schizophrenia-1
DMSO	Deoxymethylsulfoxide
DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco's Modified Eagle Medium
DR1	Dopamine Receptor 1
DTI	Diffusion Tensor Imaging
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
eGFR	Estimated Glomerular Filtration Rate
ERK	Extracellular signal–Regulated Kinases
Euro-WABB	European Wolfram, Alstrom and Bardet-Biedl syndrome study
FA	Fractional Anisotropy
FBS	Fetal Bovine Serum
FZD	Frizzled
FWE	Family-wise Error
GGT	Gamma Glutamyl Transferase
GM	Grey Matter
GLI	Glioma-associated oncogenes
GLP-1	Glucagon- Like-Peptide-1
GPCR	G Protein Coupled Receptor
GSK-3β	Glycogen synthase kinase 3β

GWAS	Genome Wide Association Studies
HIFα	Hypoxia-Inducible Factor-1α
HOMA-IR	Homeostatic Model of Insulin Resistance
HTR6	Serotonin Receptor 6
IDL	Intermediate Density Lipoproteins
IFT	Intraflagellar transport
IGF1	Insulin-like Growth Factor-1
INV	Inversin
IQ	Intelligence Quotient
JATD	Jeune Asphyxiating Thoracic Dystrophy
JS	Joubert Syndrome
KDIGO	Kidney Disease: Improving Global Outcomes
MAPK	Mitogen Activated Protein Kinase
MC3	Melanocortin-3
MC4	Melanocortin-4
MCHR1	Melanin-Concentrating Hormone Receptor 1
MDCK	Madin Darby Canine Kidney
MD	Mean Diffusivity
MDRD	Modification of Diet in Renal disease (MDRD)
METAP2	Methionine AminoPeptidase 2
MKS	Meckel Syndrome
MKKS	McKusick-Kaufman syndrome
MORM	Mental Retardation- Truncal Obesity-Retinal Dystrophy- Micropenis
mTOR	mammalian Target Of Rapamycin
mTORC1	Mammalian-Target-Of-Rapamycin-Complex-1
NAFLD	Non-Alcoholic Fatty Liver Disease
NHS	National Health Service
NICE	National Institute for Clinical Excellence
NIMA	Never In Mitosis A
NPY	NeuroPeptide Y
LCA	Leber Congenital Amaurosis
LDL	Low Density Lipoproteins
LKB1	Tumour suppressor kinase Liver Kinase B1
PBS	Phosphate Buffered Saline
pCASL	Pseudo-Continuous Arterial Spin Labelling
PCD	Primary Ciliary Dyskinesia
PC1	Polycystin 1
PC2	Polycystin 2
PCP	Planar Cell Polarity
PDGFR	Platelet-derived growth factor
PFA	Paraformaldehyde
PKD	Polycystic Kidney disease
POMC	Pro-OpioMelanoCortin
PON1	Paraoxanase-1
PP	Pancreatic Polypeptide
PPAR-γ	Peroxisome Proliferator-Activated Receptor γ
PTCH1	Patched1
PWS	Prader-Willi Syndrome
RCD	Rod-Cone Dystrophy

RD	Radial Diffusivity
RFU	Relative Fluorescence Units
RHEB	Ras-Homologue-Enriched-in-Brain
ROI	Region Of Interest
SMO	Smoothened
SLS	Senior-Loken Syndrome
SHH	Sonic Hedgehog
SSTR3	Somatostatin Receptor 3
TBSS	Tract-Based Spatial Statistics
TGF- β	Transforming growth factor β
TRKB	tyrosine kinase B
TIV	Total Intracranial Volume
URECs	Urine derived Renal Epithelial Cells
US	Usher Syndrome
VBM	Voxel-Based Morphometry
VLDL	Very Low Density Lipoproteins
WASI-IV	Wechsler Abbreviated Scale of Intelligence – Fourth Edition
WM	White Matter
10m-ISWT	10m Incremental Shuttle Walk Test

1 Introduction

Bardet-Biedl syndrome (BBS) is a rare autosomal recessive ciliopathy characterised by rod-cone dystrophy, learning difficulties, polydactyly, obesity, genital abnormalities and renal dysplasia[6]. It is a member of a family of rare genetic conditions collectively known as the ciliopathies[2], all of which have defects in the structure or function of the cilium[5]. Mutations in twenty-one genes account for up to 80% of patients with a clinical diagnosis of BBS[6]. All described BBS genes code for proteins that localise to either the cilium or basal body.

1.1 Cilia

Cilia are highly evolutionarily conserved organelles projecting from the cell surface of most cells in the human body[7]. A few exceptions exist including the clara cells of the bronchioles, white blood cells, epithelial cells in the gastrointestinal tract, and T cells[8].

The cilium is structurally continuous with the cell plasma membrane but functionally distinct providing an environment unique from the cytosol. The ciliary membrane is enriched with sterols, glycoplipids and sphingolipids. A diffusion barrier exists between the plasma membrane and the ciliary membrane correlating with the invagination of the plasma membrane adjacent to the ciliary base[9].

Cilia are broadly divided into two subtypes based on their structure and function: motile and primary cilia. More than 250 polypeptides make up the ciliary ultrastructure[10]. Primary cilia are arranged in a '9+0' configuration composed of 9 microtubule doublets arranged in a cylindrical configuration and are singularly represented on each cell (figure 1.1). In contrast, motile cilia have an additional central pair of microtubule singlets (the

'9+2' configuration), contain additional components including dynein arms and radial spokes and are usually found in multiples on a single cell.

Structurally, the cilium consists of three parts: the basal body, the transition zone and the ciliary axoneme.

The basal body is derived from the mother centriole, which consists of 9 microtubules. It is comprised of triplet microtubules, subdistal appendages and transition fibres which tether the basal body to the ciliary membrane[11].

The transition zone functions to compartmentalise the cilium from the cytosol and allows it to form distinct functions including signalling in primary cilia and motility in motile cilia. It is in the transition zone that the basal body's centriolar triplet structure converts into the microtubule doublet ciliary axoneme[11,12]. It is distinguished by the presence of transition fibres[13] and Y links and is a highly evolutionarily conserved subciliary domain[14]. The Y links are thought to provide the diffusion barrier necessary to distinguish the ciliary compartment from the cytosol[11].

The nine microtubule doublet ciliary axoneme can contain subdomains such as singlet microtubules at the distal end[11].

Motile cilia can also perform sensory tasks for example the epithelial cells environment including changes in light, flow of ligands or fluids[15]. In response to external stimuli, cilia play an integral role in a number of signalling pathways discussed further in chapter 7. The nodal cilia in the embryonic node form a notable exception to the primary cilia in that they generate a rotational movement of morphogens to specify left-right asymmetry[15]. Motile cilia generate flow to allow clearance of mucus in the trachea, movement of the sperm cell, movement of the egg cell along the fallopian tube, and

movement of cerebrospinal fluid in the brain ventricles[12]. Movement is generated by dynein arms attached to the outer axoneme[16]. Motile cilia can also perform sensory tasks for example the epithelial cells in the airways which are also chemosensory[12].

1.1.1 BBS proteins and the cilium

BBS proteins localise to the basal bodies and centrosomes in mammalian cells[17]. Many of the proteins have not been fully characterised. A subset of these proteins form a coating complex known as the BBSome which has been implicated in transport of protein cargoes from the cytosol to the cilium[18,19]. The BBSome is a stable heptameric complex formed of BBS1, BBS2, BBS4, BBS5, BBS7, BBS8 and BBS9[3]. BBS3 is a small ARF-like GTPase that localises to the basal body and ciliary membrane, interacting with BBS1 in its GTP-bound state to form a coat on the surface of membrane lipids of cellular vesicles[20]. It has been suggested that interaction with BBS3 is required for transport of the BBSome to the cilium, and that the function of BBS3 is to regulate trafficking of the BBSome through interaction with BBS1[21].

The BBSome acts as a connector linking vesicles required to be transported from the cytosol into the cilium with key proteins in the intraflagellar transport system responsible for generating and maintaining the cilium[22].

BBS6, BBS10 and BBS12[23] form another complex localising to the basal body known as the chaperonin complex. This complex is thought to assist in assembly of the BBSome by mediating the association of the BBSome with a CCT/TriC chaperonin complex that assists in appropriate folding of the BBsome[23].

CEP290 (BBS14) is a centriolar satellite protein that interacts with BBS4 allowing it to form the BBSome[22]. BBS13 localises to the basal body and is required for the formation of the primary cilium[6,24].

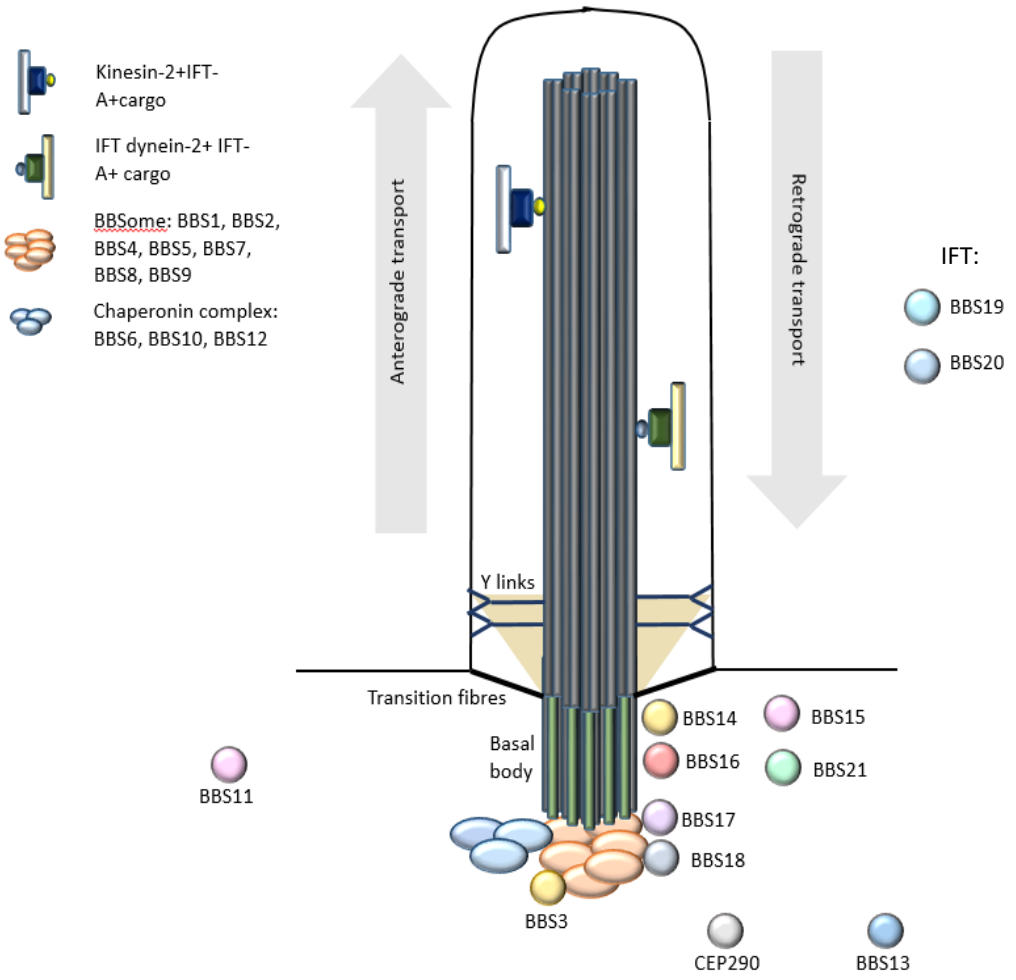


Figure 1.1 Structure of the primary cilium

BBS proteins localise primarily to the basal body. Schematic of the primary cilium demonstrating the subciliary compartments. The transition zone is depicted in yellow. The predicted localisation of known BBS proteins is depicted. Adapted from Forsythe and Beales[3] and Mitchison and Valente[2]

Figure 1.1 demonstrates the predicted localisation of all known BBS proteins and table 1.1 summarises the function of BBS proteins where this is known.

1.1.2 Intraflagellar transport

Intraflagellar transport (IFT) is a bidirectional transport system for driving cargo movement along the cilia axonemal microtubules. The cilium lacks protein synthesis and is therefore entirely reliant on transport of proteins from the cytosol[12]. The IFT system consists of at least three components: the ciliary motor proteins and IFT- complex A and complex B. IFT complexes A and B are biochemically and functionally distinct. Mutations in IFT-A proteins lead to short bulgy cilia whereas mutations in IFT-B lead to absent or very short cilia[11]. The precise function of the IFT complexes is unknown but it is thought that they act as adaptors between the IFT motors and the protein cargo.

An anterograde IFT system moves proteins along the axoneme towards the distal tip powered by kinesin motors and IFT retrograde transport takes proteins from the distal tip back out of the cilium into the cytosol, powered by dynein motors. IFT transports signalling proteins, α-/β-tubulin proteins for ciliary maintenance, as well as other proteins to the cilium[11].

1.2 The ciliopathies

The number of conditions classified as ciliopathies has increased significantly in the last 20 years[2] now including over 35 separate conditions[11].

Ciliopathies are classically divided into motile and primary ciliopathies according to the type of ciliary function affected[15]. This is broadly reflected in the clinical phenotype, although some overlap exists, for example the co-existence of motile respiratory ciliary dysfunction in patients with molecular confirmation of BBS [Personal communication with Professor Chris O'Callaghan]. This is corroborated by findings by Shah *et al* who identified that BBS proteins are expressed in motile cilia and that loss of function of BBS

proteins in mouse models leads to defective airway motile cilia and is associated with bulbous motile cilia tips filled with vesicles[25]. Other examples of overlaps between motile and primary ciliopathies exist. For examples, patients with Polycystic Kidney disease (PKD), a disease of primary cilia, have a higher incidence of bronchiectasis (a typical feature of the motile ciliopathy Primary Ciliary Dyskinesia (PCD)[26,27].

Ciliopathies can also be classified according to the localisation of the disrupted protein; First order ciliopathies include conditions resulting from the dysfunction of proteins that localise to the cilium or basal body[11] such as BBS proteins or the IFT proteins implicated in Jeune Asphyxiating Thoracic Dystrophy (JATD). Second order ciliopathies result from disruption of proteins that do not localise to the cilium or basal body but have a role in maintaining the cilium structure or function[11] . PCD caused by mutations in *DNAAF4*, an axonemal dynein assembly factor, is an example of a second order ciliopathy[11].

For practical purposes, the ciliopathies can also be loosely divided into skeletal ciliopathies and Oro-Facio-Digital (OFD) syndromes, renal ciliopathies, ophthalmological ciliopathies and multisystem ciliopathies. However, the overlap of clinical features between ciliopathies can be so significant that it can be difficult to assign a specific diagnosis. The ciliopathies can arguably be viewed as a continuum of diseases categorised by organ involvement and severity. Genes can be implicated in multiple ciliopathies with limited, or no, overlap in phenotype. An example of this is that different alleles of *TMEM231* can be associated with Meckel syndrome (MKS), OFD and Joubert syndrome (JBTS)[11].

Of the 150+ genes known to cause ciliopathies[11], there is now evidence for a broad correlation between the structural ciliary location of the aberrant protein and the associated clinical condition: BBS proteins localise primarily to the basal body, skeletal

ciliopathy genes localise to the IFT system, renal ciliopathies localise to the transition zone[2] although significant overlap exists[11].

An ongoing conundrum is how different mutations in the same gene can lead to different ciliopathies that are associated with specific functions in the cilium. Certain mutations in *IFT172*, for example can lead to a skeletal ciliopathy whereas other mutations can lead to BBS or RP[11]. This may be due to certain mutations affecting the interaction between IFT172 and other complexes such as the BBSome, rather than producing an aberrant protein[11]. Similarly, CEP290 interacts with a number of different complexes in distinct subciliary localisations including the transition zone and centriolar satellites which may account for its phenotypic variability[2].

Mutations in different isoforms of the same gene product may also be a cause of different phenotypic outcomes. In BBS3 (ARL6), for example, mutations only in one isoform are associated with retinal dystrophy[11].

It has been suggested that a subset of ciliopathy genes remain undiscovered: Only 80% of patients with a clinical diagnosis of BBS can be confirmed molecularly[28,29], and the molecular diagnostic rate is around 70% for MKS[30,31], 60% for JBTS and only 40% for JBTS with kidney involvement[32,33].

1.3 Bardet-Biedl syndrome

BBS was initially described in the 1880s by doctors Moon and Laurence, who published their findings on a family with retinitis pigmentosa, obesity and intellectual disability[34]. The individuals described in this family later went on to develop spastic paraparesis[6]. In 1920 and 1922 doctors Bardet and Biedl independently described families with retinitis pigmentosa, polydactyly and obesity[3]. From 1925 onwards the syndrome was known as

Laurence-Moon-Bardet-Biedl syndrome. Based on historical descriptions of patients with the syndrome it was later suggested that the condition was two separate entities: Laurence-Moon syndrome and Bardet-Biedl syndrome depending on the presence or absence of spastic paraparesis respectively[35]. It is now widely accepted that the syndromes are allelic and that spastic paraparesis is infrequently present and represents variability within the syndrome[35]. The condition is now known simply as Bardet-Biedl syndrome[3].

1.3.1 Epidemiology

The prevalence of BBS varies in different populations. It has been estimated to occur in 1 in 100,000 in North America and up to 1 in 160,000 in parts of Northern Europe[6,36]. It is much more common in some populations with high rates of consanguinity including Kuwaiti Bedouins (1: 13,500)[37] and in Newfoundland (1:18,000)[38]. Founder mutations have been identified in the Faeroe, Hutterite and Tunisian populations[36,39,40]. In Northern European populations two recurrent mutations are seen in a high proportion of patients, p.Met390Arg is seen in up to 80% of patients with *BBS1* mutations and P.Cys91Leufs*5 is seen in a high proportion of patients with mutations in *BBS10*[1]

1.3.2 Genetics

Around 80% of patients with a clinical diagnosis of BBS have a mutation in one of 21 known BBS genes. The remaining 20% may have an alternative diagnosis with similar clinical features, or may have mutations in BBS related genes that remain undiscovered. Table 1.1 demonstrates the known BBS genes, their predicted prevalence in the BBS population, and known or presumed location and function in the cilium.

1.3.3 Inheritance

Bardet-Biedl syndrome is inherited in an autosomal recessive manner. It has been suggested that inheritance could be triallelic[41-43], whereby a third allele is required to unfold the BBS phenotype, on the basis that some affected individuals have been identified with three mutations in two genes[41,42] whilst others with two *BBS1* p.Met390Arg mutations appeared to be unaffected[43]. Although this theory is appealing in BBS where there is significant phenotypic variability, further studies have failed to support these findings[40,44-46]. The theory is not currently in practical application in the UK national BBS clinics. It remains possible that a third mutation in a different gene could impose a modifier effect. Other ciliopathy genes have been proposed to display a modifier effect including *MKS1, MKS3, CEP290, AHI1*[19,47,48]. However, as our understanding of the complex interplay between genes continues to expand it seems more likely that a multitude of factors affect the penetrance and expression of genetic variants. It is also possible that the patients with two *BBS1* p.Met390Arg mutations who did not appear to have BBS could have delayed phenotypic expression as has been observed on a number of occasions in the national Bardet-Biedl syndrome clinics [own observations]. Variable expressivity is a characteristic feature of BBS[2]. This can in part be accounted for by variation in genotype. However, considerable variation exists even among siblings. It is possible that future analysis of '-omics' data (genomics, epigenomics, transcriptomics, proteomics and metabolomics) which investigates the complex interplay of genes, transcription, protein expression and metabolism, may yield some answers that could further clarify the phenotypic variability[35]. Projects are ongoing at the UCL Great Ormond Street Institute of Child Health to unravel the contribution of the '–omics' to variability in the BBS phenotype.

Table 1.1 BBS genes

Gene	*Frequency*	*Function*
BBS1	23%	BBSome protein
BBS2	8%	BBSome protein
BBS3/ARL6	<1%	GTPase
BBS4	2%	BBSome protein
BBS5	<1%	BBSome protein
BBS6/MKKS	6%	Part of chaperonin complex
BBS7	2%	BBSome protein
BBS8/TTC8	1%	BBSome protein
BBS9	6%	BBSome protein
BBS10	20%	Part of chaperonin complex
BBS11/ TRIM32	<1%	E3 ubiquitin ligase
BBS12	5%	Part of chaperonin complex
BBS13/ MKS1	4.5%	Centriole migration
BBS14/ CEP290/ NPHP6	1%	Basal body: RPGR interaction
BBS15/ WDPCP	1%	Basal body: localisation of septins and ciliogenesis
BBS16/ SDCCAG8	1%	Basal body: interacts with OFD1
BBS17/ LZTFL1	<1%	Negative regulator of the BBSome ciliary trafficking and sonic hedgehog pathway signalling
BBS18/BBIP1	<1%	Interacts with the BBSome
BBS19/ IFT27	<1%	Intraflagellar transport protein
BBS20/ IFT172	<1%	Intraflagellar transport protein
BBS21/ C8ORF37	<1%	Unknown function. Localises to the primary cilium in human retinal pigment epithelial cells.

Adapted from Forsythe & Beales[3] and Forsythe and Beales[6]

1.3.4 Diagnosis

BBS is a pleiotropic disorder and the diagnosis is made on a combination of features based on the seminal paper on diagnostic criteria by Beales *et al* in 1999[49]. Table 1.2 outlines the major and minor clinical criteria required to make a clinical diagnosis as well as the reported frequency of each feature. Either three major and two minor criteria are required, or four major and one minor clinical feature to make a clinical diagnosis. Approximately 80% of patients with a clinical diagnosis have molecular confirmation of their diagnosis on sequencing of the 21 known BBS genes[35]. The molecular diagnostic yield has increased considerably over the last ten years. When the UK national BBS clinics were initially commissioned the presence of only the four most common variants *BBS1* p.Met390Arg, *BBS2* p.Tyr24* and p.Arg275* and *BBS10* P.Cys91Leufs*5 were tested, delivering molecular confirmation in around 40% of patients[35]. Following the introduction of next generation sequencing gene panels the molecular diagnostic yield has increased to 80%[6]. It is likely that the remaining 20% of patients have mutations in genes that are as yet undiscovered or that the particular mutation types harboured by these patients are not detectable on the gene panels currently used for diagnostic purposes.

A further consideration is that genomics is changing the diagnostic yield thus both benefiting and complicating diagnosis. Patients have been referred to the UK national BBS clinics with molecular confirmation of BBS without fulfilling any of the hard handle diagnostic criteria. Some patients have subsequently been found to have a different syndrome [own unpublished observations]. Furthermore, major sequencing projects such as the UK 10,000 Exomes project, Deciphering Developmental Delay study (DDD) and the 100,000 Genomes Study have identified patients with known pathogenic mutation in *BBS1* where patients exhibit non-syndromic rod-cone dystrophy, sometimes

at a much later stage in life than otherwise expected in BBS [own unpublished observations]. These new findings call for a more flexible approach when diagnosing BBS in order to encompass the full disease spectrum from the severe classical form to non-syndromic rod-cone dystrophy.

Table 1.2 Diagnostic features and prevalence in BBS

Feature	Prevalence
Primary features	
Rod-cone dystrophy	93%
Polydactyly	63-81%
All four limbs	21%
Upper limbs only	9%
Lower limbs only	21%
Obesity	72-92%
Genital abnormalities	59-98%
Renal dysplasia	53%
Intellectual disability	61%
Secondary features	
Speech delay	54–81%
Developmental delay	50–91%
Diabetes mellitus	6–48%
Dental anomalies	51%
Congenital heart disease	7%
Brachydactyly/ syndactyly	46–100%/8–95%
Ataxia/ poor coordination	40–86%
Anosmia/hyposmia	60%

Adapted from Forsythe & Beales[3]

1.3.5 Clinical features

Six major clinical features define BBS: retinal degeneration, obesity, intellectual disability, polydactyly, genital abnormalities and renal dysplasia. Major features and minor features are discussed below.

1.3.5.1 Rod cone dystrophy

BBS is a major cause of syndromic retinal dystrophy[3]. The usual constellation observed in BBS is that of rod-cone dystrophy, although cases of cone-rod dystrophy are also seen in the clinics [own unpublished observations]. This feature is discussed further in chapter 4.

1.3.5.2 Obesity

Obesity is a prominent feature of BBS and is present in the majority of affected individuals[6]. Rapid weight gain usually starts within the first year and continues throughout life[3]. The distribution of adipose tissue appears diffuse in childhood but becomes primarily truncal in adulthood[3]. The molecular pathophysiology underlying the obesity is poorly understood but is further discussed in chapter 5.

1.3.5.3 Renal dysplasia

Renal disease is present in up to 53% of patients[3], but end-stage renal disease is relatively infrequent occurring in around 8% of patients[1]. Structural abnormalities extend beyond the cystic kidney phenotype usually reported in ciliopathies and is described in further detail in chapter 3.

1.3.5.4 Intellectual disability

Intellectual disability is present in 61% of patients with BBS. Most affected individuals are thought to have mild to moderate intellectual disability[38,50,51]. Additional cognitive features include slower thought processes, obsessive-compulsive disorder and high levels of anxiety[51,52]. The neurocognitive phenotype in BBS is discussed in further detail in chapter 7.

1.3.5.5 Polydactyly

Many patients with BBS are born with polydactyly which is usually postaxial in nature[49]. Cases of interdigital polydactyly are rare but have been observed in the UK national BBS clinics [own unpublished observations]. Polydactyly is thought to be a result of aberrant Sonic Hedgehog (Shh) signalling[53] and may involve all four limbs or any combination of upper and/ or lower limbs[3]

1.3.5.6 Genital abnormalities

Genital abnormalities are more frequently observed in males than females with BBS[49]. Most men with BBS present with a micropenis[49]. Genital abnormalities in women with BBS are highly variable and can present with vaginal atresia, septate vagina, hydrometrocolpos, persistent urogenital sinus and other structural abnormalities[6]. Although these structural aberrations have most frequently been described in patients with McKusick-Kaufman syndrome (MKKS)[54] they have also been seen in patients with other genotypes in the UK national BBS clinics [own unpublished observations]. Hypogonadism is most likely hypogonadotrophic in origin[3]. Relatively few individuals with BBS have had children[49]. This is likely to be due only in part to genital abnormalities. Relatively few affected individuals seem to form long term partnerships [own

unpublished observations]. Several affected men and women with BBS have had children[49].

1.3.5.7 Secondary features

A number of secondary features are supportive of a diagnosis of BBS.

1.3.5.7.1 Speech delay

Most children do not attain intelligible speech until the age of 3-4 years old. In addition it has been suggested that children with BBS often substitute consonants in the beginning of words and omit the last consonant of a word[49].

1.3.5.7.2 Developmental delay

The vast majority of children with BBS have global developmental delay. Although most attain language, gross and fine motor skills to a reasonable standard, many adults with BBS continue to struggle with social cues throughout life[49].

1.3.5.7.3 Behavioural difficulties

Many children with BBS struggle with behaviour. This often occurs in conjunction with a diagnosis of autistic spectrum disorder (ASD)[51] and includes angry outbursts and disinhibited behaviour[49]. Many individuals with BBS also struggle with emotional lability.

1.3.5.7.4 Neurological dysfunction

This has been poorly characterised in the literature. A small proportion of patients have signs of cerebellar involvement including a wide based gait, dysdiadochokinesia, truncal

hypotonia and nystagmus[49]. There is, however, no supportive evidence of cerebellar abnormalities associated with BBS. Lower limb hypertonia has also been described in a small subsection of patients[49].

1.3.5.7.5 Diabetes mellitus

Diabetes usually occurs in late adolescence or adulthood and is primarily in the form of type 2 diabetes mellitus. The prevalence of type 1 diabetes does not appear to be above the expected rate for the population[55]. Six- 16% of patients with BBS are reported to have diabetes [55,56] . Impaired glucose tolerance has also been described[6,55].

1.3.5.7.6 Brachydactyly and syndactyly

Brachydactyly is frequently observed in patients with BBS. Syndactyly is less commonly present and usually occurs between the second and third toe[49].

1.3.5.7.7 Orodental abnormalities

These include a high arched palate, hypodontia and dental crowding. Many patients with BBS require tooth extraction to overcome dental crowding[6].

1.3.5.7.8 Structural cardiovascular abnormalities

The prevalence of structural cardiac defects is unclear and is reported to be 7-50%[49,57]. Patients in the UK national BBS clinics are not routinely screened for cardiac

abnormalities. Structural defects include valve stenoses, atrial and ventricular septal defects and patent ductus arteriosis[49,58].

1.3.5.7.9 Facial dysmorphism

Affected individuals often have dysmorphic features including brachycephaly, macrocephaly, bitemporal narrowing, narrow forehead, a long smooth philtrum, short upward turned nose, retrognathia and large ears[59]. These clinical features are not always present and can be subtle.

1.3.5.7.10 Hepatic involvement

Perilobular fibrosis, periportal fibrosis with small bile ducts, bile duct proliferation with cystic dilatation, biliary cirrhosis, portal hypertension, and congenital cystic dilations of both the intrahepatic and extrahepatic biliary tract have been described[15].

1.3.5.7.11 Gastrointestinal disease

A small number of patients with BBS have been reported to have Hirschprung's disease [49,59]. Some patients also have hepatic involvement which may include cystic dilatation, fibrosis, biliary cirrhosis or portal hypertension[15].

1.3.5.7.12 Anosmia

Partial anosmia has been described in several mouse models[60,61] and previous research demonstrated that out of a cohort of 19 individuals with BBS 47% were completely or partially anosmic[6,61,62].

1.4 Managing BBS in the future

BBS is currently managed symptomatically focussed on aggressive treatment of hypertension, diabetes and obesity in order to minimise the risk of exacerbating the disease process in vulnerable organ systems, in particular the eyes and kidneys[5].

Following on from the considerable advances in our understanding of the clinical BBS phenotype as well as the significant improvements in molecular diagnostics, the expected next step will be the development of novel therapies which will target the disease pathophysiology rather than provide symptomatic relief.

The last decade has seen considerable advances in genetic therapeutic developments as well as targeted non-genetic interventions. Potential future applications for BBS are discussed here.

1.4.1 Genetic therapies

The pleiotropic nature of BBS presents a challenge in developing therapies to treat the condition as a whole. The rod-cone dystrophy seen in the majority of patients with BBS is a particularly attractive focus for developing therapies. Not only is it the disease feature patients often identify as the most debilitating part of their condition, it also offers a number of practical advantages namely the presence of a control (other) eye, relatively easy access and, most often, a treatment window where the retina is relatively healthy before deterioration sets in[35,63].

Other major features of BBS have received limited efforts in terms of developing genetic therapies. Renal dysplasia presents a particular challenge as structural aberrations are

usually prenatal in onset, and the natural history and phenotypic diversity of renal disease is poorly understood[35]. Furthermore, renal disease can be both primary in nature or secondary to hypertension or diabetes mellitus which are both more frequently observed in BBS than in the general population[35].

For future development of therapies for BBS, new disease models are being developed and are complementing and reducing the need for traditional animal research models. *In vitro* organ systems are being generated from induced pluripotent stem cells[63-68]. Cells derived from adults are reprogrammed into stem cells and can subsequently be differentiated into an array of cell types through the addition of growth factors[66]. A diverse selection of cells have been reprogrammed including dermal fibroblasts[69], renal epithelial cells[70], keratinocytes[71] and peripheral blood cells[72].

Urine derived renal epithelial cells is another emerging model system that has been used to model the ciliopathy Joubert syndrome[73,74]. This is a particularly attractive option as it is entirely non-invasive and offers an organ-specific model. Work using this system is described in chapter 7. The four genetic therapies most likely to benefit patients with BBS include gene therapy, readthrough therapy, exon skipping therapy and genome editing.

1.4.1.1 Gene therapy

Gene therapy has been developed for a number of conditions and several ciliopathy-related eye diseases have demonstrated good outcomes on gene replacement including Usher Syndrome (US) and Leber Congenital Amaurosis (LCA)[61,75,76].

Gene therapy involves integrating a wild-type gene of choice into the host genome with the help or a viral or non-viral vector. The type of vector, as well as gene size, affects the likelihood of success. The best results are achieved in conditions where only a small amount of expressed gene product is required to deliver an acceptable phenotypic effect[77]. Figure 1.2A demonstrates how gene therapy for BBS could work.

Retinal gene therapy requires invasive ophthalmic surgery, hence the aim is to generate therapy for BBS with a lasting effect, thus avoiding frequent surgical interventions. The recent launch of Luxturna, which provides gene therapy for RPE65-associated LCA, is encouraging. It is the first gene therapy for eye disease that has received FDA approval. It is noteworthy that extensive optimisation has been required prior to FDA approval to ensure the safety and efficacy of Luxturna[63,78,79]. Gene therapy for *BBS1* is underway. Recent published work using knock-in mouse models with the common *BBS1* p.Met390Arg mutation demonstrated that gene therapy could be successfully delivered to the target tissue using viral AAV vectors containing the wild type *Bbs1* construct[80]. This rescued both BBSome formation and rhodopsin localisation. Trends towards better electroretinograms are encouraging[80].

These developments are inspiring, but several challenges exist, including avoiding the generation of an immune response, developing safe and effective vectors, avoiding gene overexpression and any resultant cell toxicity as well as considerations regarding optimum timing in the natural history of retinal dystrophy to develop gene therapy[61,80,81].

1.4.1.2 Read through therapy

Nonsense mutations account for approximately 11% of the total mutational load in BBS[5]. Readthrough therapy exploits imperfection in the genetic proof-reading system at the level of RNA translation[82]. Ordinarily, RNA from premature stop codons is subject to nonsense mediated decay[83]. Readthrough therapy works by destabilising the translational ribosome allowing it to ignore premature stop codons and insert a near-cognate amino acid in its place (figure 1.2B). The result is a full length protein which, depending on the significance of the site of the mutation, may deliver some or all of the wild-type protein function[84]. Pre-clinical trials using readthrough therapy in a number of ciliopathies include trials on PCD[85], Usher syndrome[86], and RP2 Retinitis Pigmentosa[87]. Readthrough therapy in clinical trials have been completed in Cystic Fibrosis[88,89] and Duchenne Muscular Dystrophy with promising results[90,91].

1.4.1.3 Exon skipping therapy

Exon skipping therapy works by allowing mutated exons to be 'skipped' during transcription. As a result, a truncated RNA product is produced resulting in a truncated protein retaining the products of the remaining exons[92,93] (figure 1.3A).

Oligonucleotides are designed to specific exons/ introns of interest, often by targeting exonic splice enhancers. This form of genetic therapy is often used to target mutations that disrupt the genetic reading frame. Exon skipping therapy has not been explored in BBS since relatively few patients (9%)[5] would be eligible according to their genetic mutation(s). Frameshift mutations are most commonly seen in patients with *BBS10* but this is not a candidate for exon skipping as it only contains two exons. The remaining frameshift mutations in the UK BBS population are overwhelmingly private (unique) and

therefore require truly individualised therapy. Exon skipping therapy has reached pre-clinical trials for both LCA[92] and US[94], and has been developed to the level of clinical trials for Amyotrophic Lateral Sclerosis[95] and Duchenne Muscular Dystrophy (DMD)[96].

1.4.1.4 Genome editing

Genome engineering has the potential to correct a vast array of genetic aberrations as it allows DNA to be replaced, deleted or corrected[97]. Molecular 'scissors' in the form of endonucleases, create targeted double stranded DNA breaks allowing for restoration to the wild type genotype through DNA repair[97] (figure 1.3B). Not only could this potentially deliver therapies for the future, it can also be used *ex vivo* to create human model systems which eliminate genetic background noise by comparing control model systems to control model systems genetically engineered to contain a genetic aberration of interest[35].

Genome editing has not yet reached clinical trials in treating ciliopathies but initial results in cells from patients with the motile ciliopathy PCD indicate that delivery of the wild-type *DNAH11* gene can restore its function at the cellular level[98]. Clinical trials assessing the effect of gene editing in treatment resistant Leukaemia[99] and Hypertrophic Cardiomyopathy caused by *MYBPC3* mutations[100] have demonstrated the potential of gene editing in treating human disease. There are significant challenges to overcome before genome editing can be considered a likely adjunct to other therapies in BBS. Off-target effects, whereby endonucleases erroneously target unintended DNA sequences as well as the intended sequence, are a particular concern in developing genome editing for clinical purposes. Considerable optimisation efforts must be made in order to deliver safe, efficacious and specific therapies[101].

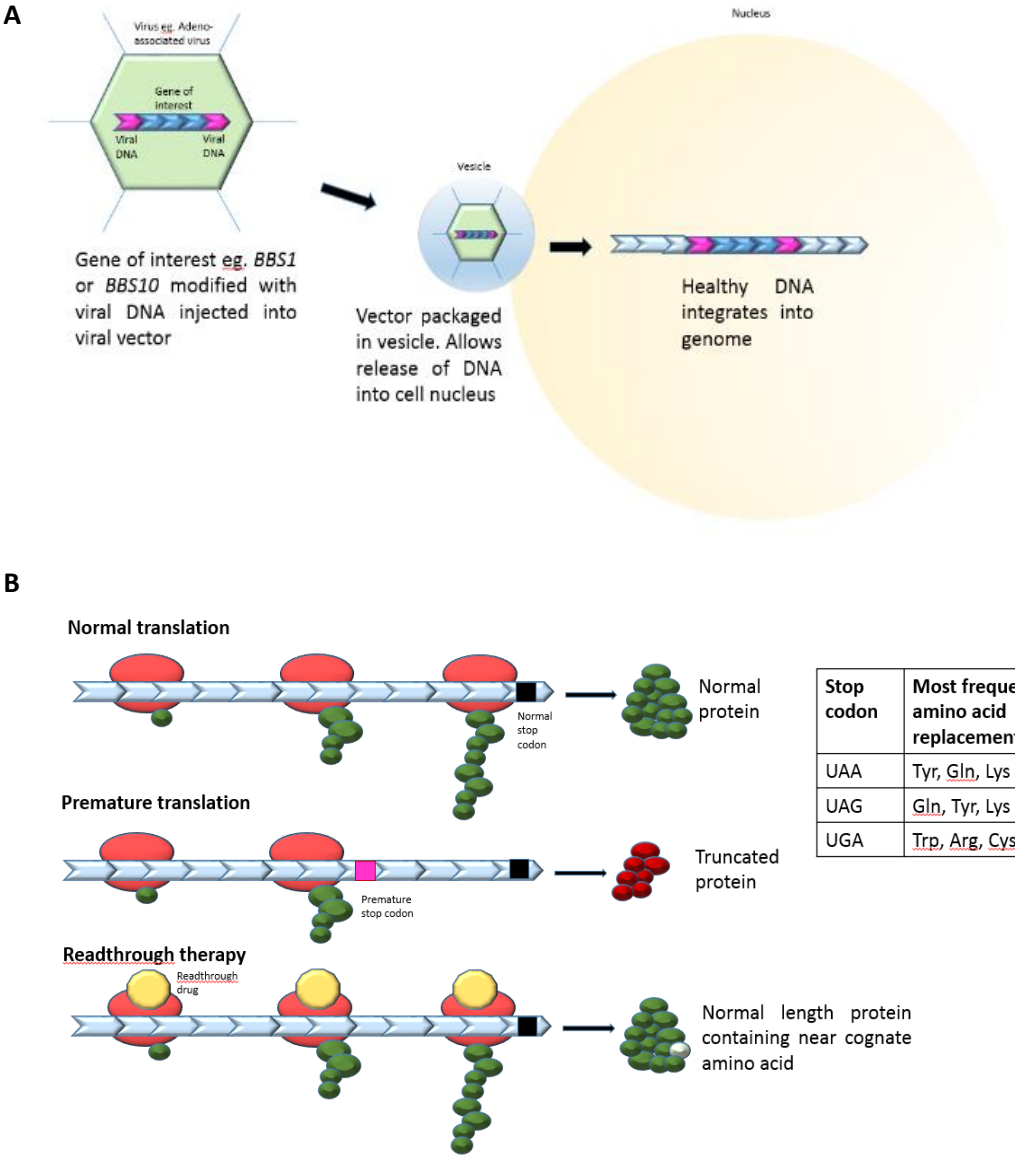


Figure 1.2A and B. Prospective personalised therapeutics for Bardet-Biedl syndrome

Several genetic therapeutic modalities will likely be employed. Percentages based on mutations observed in UK BBS patients (n=265). 1a. 70% of patients could benefit from gene therapy for patients with mutations in *BBS1* and *BBS10*. Currently animal trials are underway for *BBS1* gene therapy. 1B. 11% of patients could benefit from premature termination codon therapy. The sequence of the premature stop codon determines the likely substituted amino acid (presented here in order of frequency). The most frequently amino acid replacements are reported here. Figure adapted from Kenny *et al*[5]

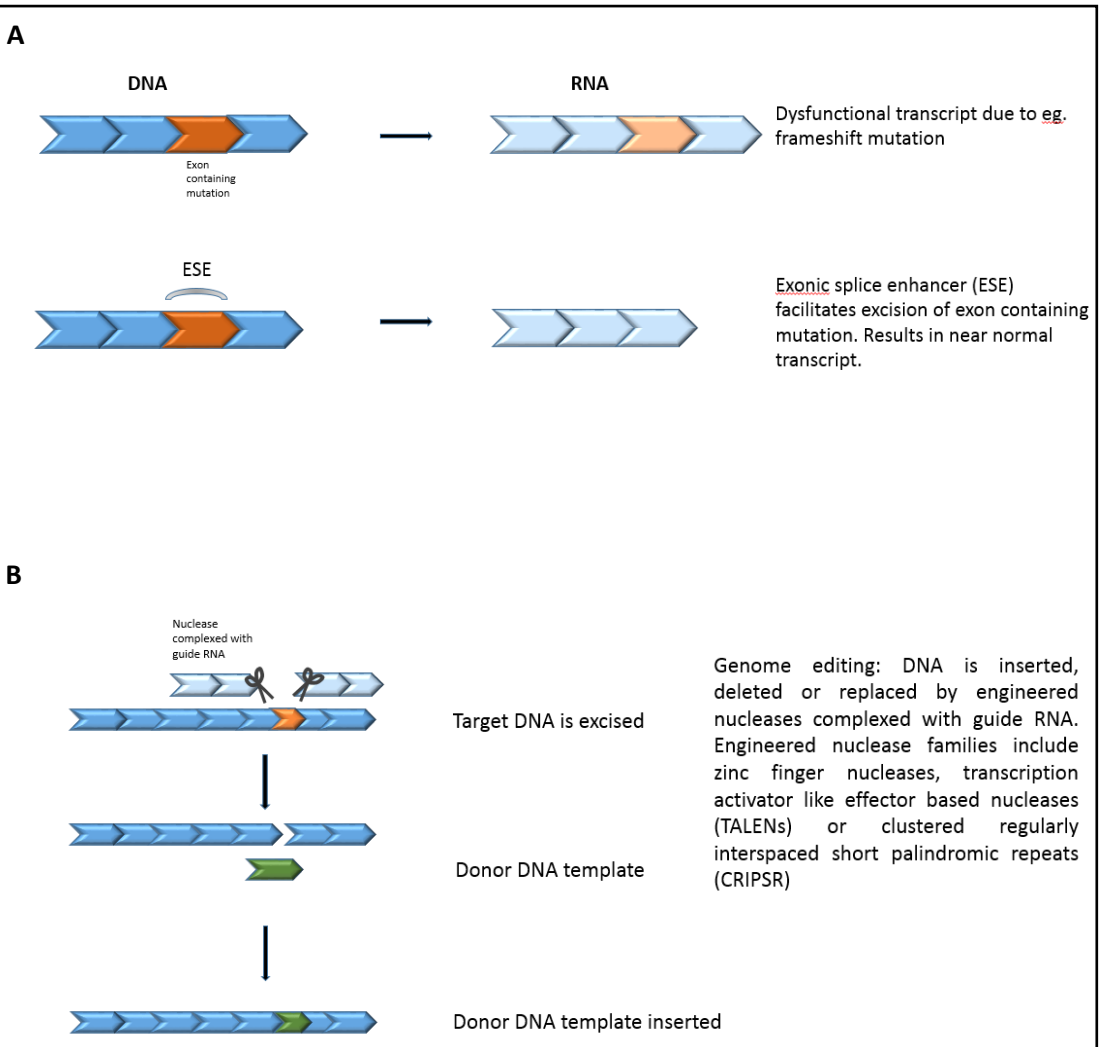


Figure 1.3A and B. Prospective personalised therapeutics for Bardet-Biedl syndrome

Several genetic therapeutic modalities will likely be employed. Percentages based on mutations observed in UK BBS patients (n=265). 1C. 9% of patients could benefit from exon skipping therapy. D. The remaining 16% of patients have primarily private mutations which are not amenable to any established genetic therapeutic approaches. A promising approach includes genome editing. *Some patients in this group may benefit from one of the other therapeutic interventions. Figure adapted from Kenny *et al*[5]

1.4.2 Non-genetic therapies

Non-genetic approaches to therapy should also be considered in managing BBS in the future. As an adjunct to traditional management of the considerable morbidity burden seen in BBS, this includes considering pharmacological as well as non-pharmacological management options to improve the overall wellbeing and quality of life.

1.4.1.5 Targeted therapies

Therapies targeting the downstream effects of aberrant BBS signalling have not previously been explored, most likely because the effects of BBS mutations in cell signalling are only beginning to come to light.

BBS mutations have previously been demonstrated to cause leptin resistance hence disrupting the hypothalamic leptin-melanocortin pathway[102] and leading to obesity. Evidence from *Bbs* knockout mice demonstrates that intravenous administration of a melanocortin receptor agonist decreases body weight and food intake[103]. In humans a recent clinical trial with Melanocortin-4 Receptor agonist Setmelanotide in BBS and Alström Syndrome as well as other forms of syndromic obesity has shown promising results[104].

1.4.1.6 Drug repurposing

Given the cost and safety issues involved in developing new pharmaceutical therapies the possibility of drug repurposing, whereby drugs that are already FDA approved for one purpose are used to treat a different condition, is an attractive and economical option[35]. Preliminary work investigating the effect of Rapamycin on the *Bbs* zebrafish

renal phenotype indicated that the drug may, in part, rescue the phenotype[59], however, no further supporting evidence has been published.

1.4.1.7 Pharmacogenomic profiling

Patients with BBS often have complex medical requirements and as a result are particularly vulnerable to the adverse effects of polypharmacy. Pharmacogenomic profiling whereby the effect of the genome and genetically inherited mutations on drug response is predicted is an attractive prospect for this patient group[5,35,105]. Several companies offer this on a private basis. The evidence base for many of the privately offered tests is unclear but there is increasing interest in incorporating pharmacogenomics profiling in the National Health Service (NHS) delivery and this is likely to become available in the coming years[35]. Figure 1.4 summarises different potential management options for BBS as well as the current stage of development.

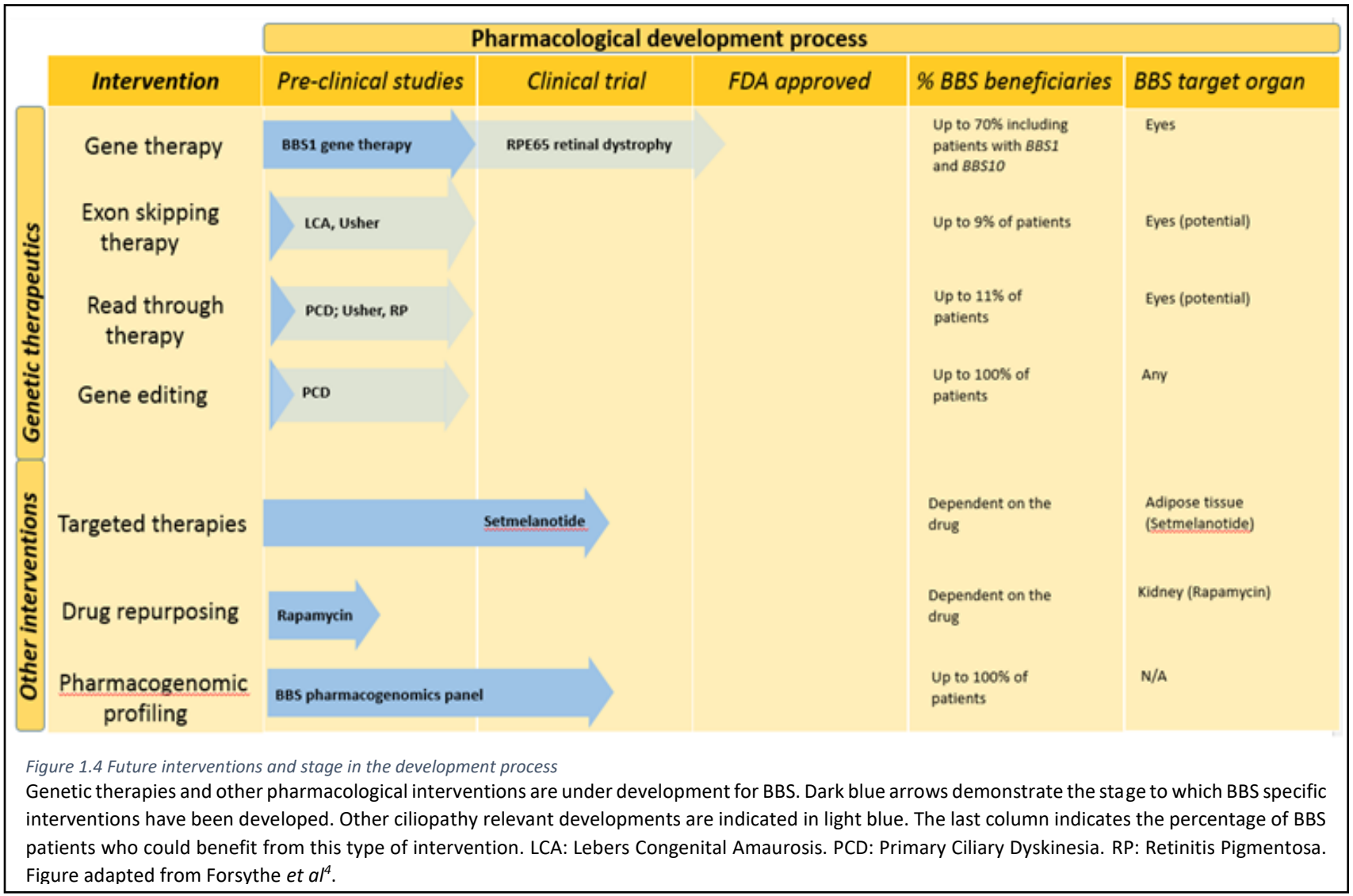


Figure 1.4 Future interventions and stage in the development process

Genetic therapies and other pharmacological interventions are under development for BBS. Dark blue arrows demonstrate the stage to which BBS specific interventions have been developed. Other ciliopathy relevant developments are indicated in light blue. The last column indicates the percentage of BBS patients who could benefit from this type of intervention. LCA: Lebers Congenital Amaurosis. PCD: Primary Ciliary Dyskinesia. RP: Retinitis Pigmentosa. Figure adapted from Forsythe *et al*[4].

1.4.1.8 Lifestyle modification

Lifestyle choices have previously been overlooked as potential modifiers of genetic disease but are increasingly being recognised as important modifiers in other genetic diseases such as PKD where physical activity has been identified as a potential therapeutic intervention to slow decline in kidney function[106]. The effect of exercise on cognition in patients with BBS is discussed in chapter 8.

1.5 Thesis Rationale

Three decades ago very little was known about the BBS phenotype. Patients, carers and clinicians alike had limited available information on what to expect in terms of morbidity and mortality, and no access to expert advice.

Since then, there has been an exponential increase in our ability to diagnose BBS; Clinically with the publication of the seminal paper formulating the criteria for diagnosis by Beales *et al* in 1999[49]- and, molecularly, following discovery of the first BBS gene in 1994[107]. This has culminated in 2018 with the description of disease-causing mutations in 21 known genes accounting for 80% of patients with clinical features of BBS. These diagnostic developments have occurred in concert with the general diagnostic watershed in rare diseases following the integration of whole exome and genome sequencing to clinical services.

I have been part of the UK national BBS clinics since their inception in 2010. During this time around 500 people with BBS have been seen nationally, and common concerns and unmet needs from patients, parents and carers have emerged.

The overarching aim of this thesis was to answer scientific questions which genuinely reflect patient values based on what I have heard and seen over the last eight years as a clinician and researcher. My aim was to capture patients' unmet needs to the fullest and focus my research on outcomes of benefit to patients.

From the moment a parent receives the life-changing diagnosis of BBS I found that these needs and their questions can be broadly categorised into six groupings, as follows:

- *"Will my child develop kidney disease? If so when? Could they die from it? Can you treat it?"*
- *"When will my child go blind and when will there be a cure for blindness in BBS?"*

- *"Why can I not stop my child from gaining weight - will there ever be a treatment for it?"*
- *"Do people die earlier as a result of BBS?"*
- *"Are therapies are on the horizon for BBS?"*
- *"What can I do now to help my child?"*

1.6 Hypotheses

i. Patients with missense mutations and mutations in *BBS1* have a less severe renal phenotype than patients with truncating mutations and mutations in other genes (Chapter 3).

ii. Patients with missense mutations and mutations in *BBS1* have a less severe eye phenotype than patients with truncating mutations and mutations in other genes (Chapter 4).

iii. Children with BBS have healthier BMI scores than their adult counterparts (Chapter 5).

iv. BMI increases with age in adults with BBS in line with the general population (Chapter 5).

v. Patients with missense mutations and mutations in *BBS1* have a less severe obesity phenotype than patients with truncating mutations and mutations in other genes (Chapter 5).

vi. Patients with missense mutation and mutations in *BBS1* have fewer cardiovascular risk factors than patients with truncating mutations and mutations in other genes (Chapter 6).

vii. Fibroblasts and urine-derived renal epithelial cells from patients with BBS have: a. Shorter, less abundant cilia; b. An abnormal proliferation profile and c. Aberrant cilia signalling gene expression (Chapter 7).

viii. Aerobic exercise induces hippocampal volume increase in patients with BBS as assessed by voxel-based morphometry (Chapter 8).

1.7 Aims

The questions posed by the patients are addressed in turn, in the six results chapters that follow. Re-phrased from lay language, questions from affected families broadly address phenotypic variability and the natural history of BBS as well as the next frontier in rare disease research: Therapeutic intervention. The thesis is divided into three parts, addressing these specific areas/ aims:

1. Section I (Chapters 3-6): Explore the phenotype and natural history of aspects of the condition that BBS patients report as highest ranking impediments to their daily lives including rod-cone dystrophy, renal disease, obesity and cardiovascular disease. I explore these with a view to developing an in depth understanding of these phenotypes and providing preliminary data, which can help inform future therapeutic interventions.
2. Section II (Chapter 7): Assess the BBS mutant cellular phenotype, to identify potential cellular markers of disease, which could be harnessed as biomarkers of therapeutic effect.
3. Section III (Chapter 8): Explore the therapeutic effect of an exercise intervention on cognition as an intervention that BBS patients, patients and carers can

implement now and as a stepping stone for BBS research to the therapeutic era in rare disease.

2 Methods

The majority of the work done for this thesis was completed independently. Assistance is acknowledged where appropriate. Chapter 8 (Exercise and cognition study) required a multidisciplinary approach. I conceived, planned and designed the study, recruited patients and multidisciplinary collaborators, collated and interpreted the data. The fitness methods and results are the product of collaborations with physiotherapists Mr Phillip Harniess and Dr Sean Ledger. The imaging methods and results were completed by Professor Torsten Baldeweg, Professor Chris Clark, Dr Kiran Seunarine and Dr Patrick Hales. The MR imaging was done at Great Ormond Street Hospital MRI department, supervised by Mrs Tina Banks. The memory and cognition methods and results were completed by Dr Sarah Buck. I supervised two UCL psychology MSc students, Miss Holly Clegg and Miss Ming-Lei Lin, who assisted in completing the memory and cognition studies. Miss Kathryn Sparks provided specialist BBS nursing support. Mrs Sarah Flack provided dietetics support.

2.1 Patients

All patients were recruited from the national BBS clinics. Recruitment for different studies occurred at different time points. As a result, overlapping patient groups have been recruited to different studies included in this thesis.

Patients were predominantly of Caucasian and South East Asian origin and referrals were made primarily from clinical geneticists and ophthalmologists in the United Kingdom as well as from BBS UK- the British national support group.

2.1.1 Patients recruited to the renal phenotype and natural history study (chapter 3)

Patients who attended the UK national BBS clinics in the time period 2010-2014 were recruited for the study examining the phenotype and natural history of renal disease in BBS. In total, 350 patients were recruited and the following renal parameters were ascertained retrospectively: known history of renal disease, diabetes, obesity, hypertension, results of renal sonography (if available), and relevant blood and urinary tests completed following a six hour starvation period. Estimated Glomerular Filtration Rate (eGFR) was calculated according to the Schwarz-Haycock formula (height (cm) x 31/ creatinine (µmol/l)) for the paediatric population and according to the Modification of Diet in Renal disease (MDRD) formula in adults patients as this is in common use for patients in the general population with obesity and diabetes[18]. Adults were classified as hypertensive if they had a blood pressure measurement in excess of 140/90 mmHg or over 130/80 mmHg in the presence of albuminuria. Patients were included in the study if they had clinical and molecular confirmation of their diagnosis.

2.1.2 Patients recruited to the eye phenotype and natural history study (Chapter 4)

Patient surveys were sent by letter or email in 2013 to all members of the BBS patient support group and made available in the national BBS clinics to any other patients wishing to participate. A total of 400 patients had the opportunity to take part in the survey and 235 opted in.

Patient responders were included if they had a known genotype on BBS mutation analysis. The following markers of rod-cone visual deterioration were ascertained from a self-reporting patient survey: age at onset of night blindness, age at diagnosis of rod-cone dystrophy and age at registration of blindness.

2.1.3 Patients recruited to the 'Obesity phenotype and natural history study' (Chapter 5)

Patients participating in this study attended the UK national BBS clinics in London during a six year period (2010-2016). Patient data were assessed separately for children and adults reflecting differential anthropomorphic measurements and dietetics input. Patients were included in the study if they had both clinical and molecular confirmation of BBS. Only patients over the age of two were included in the study since BMI-SDS is unreliable in children who are under the age of two. For weight loss assessment on successive clinic visits only those with a BMI/ BMI-SDS outside the normal range were included. Seventy-two children with molecularly confirmed BBS were included in the study. One hundred adult patients had a recorded BMI at first visit to the national BBS clinics. BBS mutation analysis, age, weight, height, BMI/ BMI-SDS scores were ascertained for all patients at the first and each successive visit.

2.1.4 Patients recruited for the cardiovascular phenotype and natural history study (chapter 6)

Two hundred and thirty nine adult patients attending the national BBS clinics in London and Birmingham during a four year time period (2010-2014) were recruited to the study. Each patient record was assessed for height, weight, absence/ presence of hypertension and BBS mutation analysis. Biochemical profile included full blood count, renal function, liver function, inflammatory markers, endocrine and lipid profile. Clinical cardiometabolic risk factors/ co-morbidities were ascertained retrospectively from the notes including the presence of treated or untreated hypertension, hyperlipidaemia or diabetes. Patients were classified as hypertensive if they had a blood pressure over 140/90 mmHg or over 130/80 mmHg in the presence of albuminuria. The presence of structural or functional renal aberrations and cardiac structural defects was also noted.

2.1.5 Patient recruitment for the cellular phenotype study (chapter 7)

Fifty –four patients attending the adult UK national BBS clinics at Guy's Hospital gave informed written consent to having skin biopsies taken. Forty-five patients donated urine for the purposes of growing human Urine derived Renal Epithelial Cells (URECs). Adults provided informed written consent and children assented where possible with parents/ guardians consenting on their behalf. Only samples from patients with known BBS genotypes were included in the study.

Clinical parameters were ascertained on patients from whom URECs were successfully cultured including eGFR as well as the presence and description of any structural renal abnormalities.

2.1.6 Patient recruitment and study overview: 'Examining the effect of exercise on the hippocampus' (Chapter 8)

Participants were invited on the basis of confirmed BBS genotype and attendance at the UK national BBS clinic at Great Ormond Street Hospital in London, UK. Patients were excluded from the study if they were unable to comply with exercise and memory testing instructions. Patients were randomised to either the intervention (11 participants) or control groups (five participants) with adjustment to ensure an even distribution of age, gender and genotypes (*BBS1* versus other genotypes; reflecting the UK genotype distribution). All assessments were carried out at Great Ormond Street Hospital. Each assessment consisted of an exercise test with a specialist paediatric research physiotherapist, a memory and intelligence assessment and an MRI brain scan. Assessments were completed before the start of the intervention/control period and as soon as possible after the end of the intervention/control period. For logistical reasons

it was not possible to complete assessments in the same sequence (e.g. fitness assessment, followed by MRI followed by IQ and memory testing).

2.2 Study approvals

Ethical approval for skin biopsies was granted as part of the Euro-WABB (European Wolfram, Alstrom and Bardet-Biedl) study (The National Research Ethics Service West Midlands Committee- Staffordshire, REC reference 11/WM/0127). All other studies referred to in this thesis were approved through GOSH Research Ethics Committee. Project name: Molecular Genetics of Human Birth Defects – mapping and gene identification. (REC reference: 08/H0713/82). The study protocol for the Exercise study (Chapter 8) was approved by the Queen Square Research Ethics Committee (Rec reference 09/H0716/6). Research Ethics committee project name: Improving cognition in Bardet-Biedl syndrome through physical activity: neuroimaging study. The studies were conducted in accordance with the guidelines of the Declaration of Helsinki. All participants gave informed written consent prior to inclusion in studies.

2.3 Mutation analysis

BBS mutation analysis was done in stages by Dr Bethan Hoskins, Molecular Genetics department at Great Ormond Street Hospital. From 2010 until 2012 mutation analysis consisted of sequencing of the four most common mutations in BBS: *BBS1* p.Met390Arg, *BBS2* p.Tyr24*, *BBS2* p.Arg275* and *BBS10* p.Cys91Leufs*5. Where only one mutation was found, full sequencing of the relevant gene was performed to identify a second mutation. Between 2012 and 2016 mutation analysis was undertaken on all new patients and those without molecular confirmation through the UK national BBS gene

panel which encompasses 11 BBS genes including *BBS1- BB10* and *BBS12,* as well as two BBS associated genes *MKS1* and *ALMS1*. From 2016 onwards all new patients and those without molecular confirmation had mutation analysis on an extended BBS gene panel including *BBS1-BBS21* and *ALMS1*.

2.4 Skin biopsies

A 3mm shallow skin biopsy was taken from the upper or lower arm using a punch biopsy under local anaesthetic (1% lignocaine). The site was then closed with steri-strips. The samples were stored at 4˚C in Dulbecco's Modified Eagle Medium (DMEM-Glutamax; Invitrogen) supplemented with 20% Fetal Bovine Serum (FBS, Gibco). Informed verbal and written consent was obtained prior to the skin biopsy procedure.

2.5 Urine sampling

Urine was obtained via the paediatric and adult UK national BBS clinics at Great Ormond Street and Guy's Hospitals. Patients were invited to participate if they were able to provide a clean mid-stream urine specimen. Only samples from patients with clinical and molecular confirmation of the diagnosis are reported in this thesis. Patient samples were compared to samples from age and gender matched controls.

2.6 Fibroblast cell culture

Skin biopsies were divided into 4-8 pieces and placed in a sterile 25cm^2 flask. The pieces were allowed to attach on a dry surface in the sterile 25cm^2 flask for 20 minutes. Subsequently 5ml of medium (DMEM supplemented with 20% FBS and 1% Penicillin/Streptomycin (Gibco) was added slowly to avoid detachment of cells. Medium

was replaced every 3-4 days. Cells were maintained and expanded in DMEM supplemented with 20% FBS and 1% penicillin/streptomycin. In experiments assessing the number of ciliated nuclei and cilia length, cells were grown to confluency and serum starved for 48 hours in DMEM supplemented with 0.5% FBS. All cells were incubated in a humidified incubator at 37˚C and 5% CO_2. Passaging and harvesting was carried out once confluency of at least 95% was reached. Cells in a 25cm^2 flask were washed twice in 5ml Phosphate Buffered Saline (PBS) solution (Sigma-Aldrich) prior to incubation with 1ml 0.05% Trypsin/ Ethylenediaminetetraacetic Acid (EDTA, Invitrogen) until cells were detached. Neutralisation was achieved by adding 2ml DMEM to the solution. Cells were subsequently pipetted into 15ml falcons and spun in a centrifuge at 1000 rpm for five minutes. The supernatant was subsequently discarded and cells resuspended in standard medium as outlined above.

In order to ascertain the effect of Purmorphamine on fibroblast cilia, selected cell cultures were incubated for 24 hours with 20µM Purmorphamine for the last 24 hours of the serum starvation period in the usual starvation medium.

For storage, cells requiring freezing were re-suspended in a 10:1 vol/vol solution of DMEM and Deoxymethylsulfoxide (DMSO; Sigma-Aldrich), cryopreserved using a Mr Frosty freezing container (Thermo- Fisher Scientific) for 24 hours and subsequently placed in liquid nitrogen for long term storage.

2.7 Human urine derived renal epithelial cell culture

Urine samples were collected in a sterile container were stored at 4˚C and processed within four hours of collection.

Cells were isolated from the samples using a two-step centrifugation and washing protocol (full details in Ajzenberg *et al* (2015)[73]) and plated in one well of a 24 well plate in 1ml renal epithelial primary medium (1:1 Dulbecco's Modified Eagle's medium (DMEM), Ham's F12 Nutrient Mix containing 1xREGM SingleQuots, growth factors (Lonza), 10% Fetal Bovine Serum (FBS), Penicillin/Streptomycin (100µ/ml) and Amphotericin B (100µ/ml) (Invitrogen). An additional 0.5ml of primary medium was added at 24 hours, 48 hours and 72 hours after cell seeding.

Three days after seeding the primary medium was replaced with 1ml proliferation medium (REBM Basal Media (Lonza) containing 1xREGM SingleQuots and growth factors (Lonza) which selectively supports proliferation of renal epithelial cells. Proliferation medium was replaced six times per week. Once confluence levels of 80-90% were reached the cells were passaged (typically after 9-21 days).

After cell culture was established cells were seeded in 12 well plates for RNA extraction and in Nunc Lab-Tek II Coverglass 8-chambered (Sigma) for 3D culturing. A cell suspension of 100µl containing 20,000 cells was mixed with 80µl liquid Matrigel (Corning; growth factor reduced) and transferred into an incubator to allow the mixture to gel. Subsequently 300µl proliferation medium was added gently without disrupting the gel. 150µl of the proliferation medium was replaced daily throughout the culturing period (5-10 days). This work was done in collaboration with Dr Katia Nazmutidnova, and assisted by Dr Rasha Saleh and Mr Tiago Mendes.

2.8 Immunocytochemistry for human fibroblasts

Cell cultures were cultured on coverslips and washed with phosphate buffered saline (PBS). Cells were fixed in ice cold methanol 100% for five minutes at room temperature

and subsequently washed three times in PBS Tween 0.1% (Sigma-Aldrich). Cells were blocked with 1% inactivated goat's serum (Invitrogen) in PBS for one hour at room temperature and subsequently incubated for 1 hour with primary antibodies diluted at a concentration of 1:500 (acetylated tubulin and gamma tubulin) or 1:100 (Arl13B) at room temperature. Incubation at room temperature for 45 minutes with secondary antibodies conjugated to Alexa Fluor 488 or 568 at concentration 1:500 in PBS was followed by DAPI staining and mounting in Pro-long gold (Invitrogen). Images were obtained using the Zeiss AxioCam microscope system. Details of primary and secondary antibodies used here for immunodetection are outlined in tables 2.1 and 2.2. To minimise ascertainment bias at least 30 random fields of view were assessed for each experiment. Each experiment was repeated in triplicate. Confluence was assessed as an index consisting of the number of nuclei observed in an image multiplied by the magnification. The assessment of confluence was automated using a macro in ImageJ software which counts the number of nuclei per image (courtesy of Dr Dale Moulding).

Table 2.1 Primary antibodies for fibroblasts

Antibody	***Supplier***	***Catalogue number***	***Concentration***
Gamma tubulin (mouse)	AbCam	T6557	1:500
Acetylated tubulin (mouse)	Sigma-Aldrich	T6793	1:500
ARL13b (rabbit)	Protein-tech	17711-1-AP	1:100

Table 2.2 Secondary antibodies for fibroblasts

Antibody	***Supplier***	***Catalogue number***	***Concentration***
568 Donkey-anti-mouse	Life Technologies	A110037	1:500
488 Goat-anti-rabbit	Invitrogen	A11008	1:500

Once 3D cultures were ready for staining the proliferation medium was removed and cells were washed three times in Dulbecco's PBS supplemented with Calcium and Magnesium (Gibco). Cultures were then fixed using 4% paraformaldehyde (PFA, Sigma-Aldrich) for 45 minutes on a low–speed shaker. Cells were then permeabilised with a buffer consisting of PBS with 7mg/ml gelatine from porcine skin (Sigma-Aldrich) and 0.5% Triton-X-100 (Sigma-Aldrich) for 30 minutes. Cells were incubated overnight with primary antibodies (table 2.3) at 4˚C on a shaker. Cells were subsequently washed three times for ten minutes with permeabilisation buffer and twice with PBS before being stained for 45 minutes with secondary antibodies (table 2.4). After two PBS washes, cells were stained for 20 minutes with a DAPI/PBS solution (Roche; 1mg/ml stock solution diluted in 1:20,000 PBS). This was followed by a final wash with PBS. Cells were mounted with Fluoromount-G (Southern Biotech). Images were taken using a confocal microscope (LSM710 Zeiss Confocal). Cilia length measured in 3D was done using the Imaris software (Bitplane, South Windsor, CT). This work was done in collaboration with Dr Katia Nazmutidnova, and assisted by Dr Rasha Saleh and Mr Tiago Mendes.

Table 2.3 Primary antibodies for URECs

Antibody	***Supplier***	***Catalogue number***	***Concentration***
β-Catenin (rabbit)	Invitrogen	AHO0462	1:100
Acetylated tubulin (goat)	Sigma-Aldrich	T7451	1:100
ARL13b (mouse)	Protein-tech	17711-1-AP	1:100

Table 2.4 Secondary antibodies for URECs

Antibody	***Supplier***	***Catalogue number***	***Concentration***
568 Donkey-anti-mouse	Life Technologies	A110037	1:100
488 Goat-anti-rabbit	Invitrogen	A11008	1:100
680 Donkey anti-goat	Invitrogen	A10043	1:100

2.9 Cell proliferation

Proliferation was determined by a Resazurin assay (AlamarBlue Cell Viability Assay, Thermo-Fisher). Resazurin is a weakly fluorescent compound which functions as a reduction-oxidation indicator of cell viability, turning fluorescent pink on reduction indicating detection of metabolic activity.

Cells for proliferation assessment were grown in 96 well plates. Resazurin was diluted 1:10 with fibroblast growth medium and 150µl added to each well and incubated at 37˚C for 4 hours before determining cell viability according to the manufacturer's instructions. Fluorescence was registered using a BMG FLUOstar OPTIMA microplate reader and analysed using the Optima Analytics software. All results were expressed in Relative Fluorescence Units (RFU).

Samples for cell proliferation were selected based on availability and genotype severity with the expectation that this would be reflected in the proliferation phenotype. Control samples, *BBS1* p.Met390Arg samples and *BBS10* samples were selected for the assay. Each sample was done in triplicates and the mean represented.

2.10 RNA extraction

RNA was extracted from fibroblasts and hURECs grown in a monolayer to at least 95% confluence using the RNeasy kits with on-column DNA digestion (Qiagen) according to the manufacturer's instructions and RNA concentration was determined spectrophotometrically using a Nanodrop ND-1000 at ʎ=260nm/280 and 260nm/230nm. Following RNA extraction samples were stored at -80˚C.

2.11 cDNA production

cDNA was synthesised using the RT^2 First Strand kit (Qiagen). 0.5 µg total RNA was reverse transcribed for each sample according to the manufacturer's instructions including both the genomic DNA elimination step and reverse transcription. Where samples required storage prior to real-time PCR they were transferred to a -80˚C freezer.

2.12 Quantitative PCR and analysis

Gene expression of pathways directly implicated in the structure and function of primary cilia was examined via the human Primary Cilia pathway RT^2 profiler PCR array (PAHS-127Z; Sabioscience) using cDNA derived from fibroblast and URECs from BBS patients with molecular confirmation of the diagnosis and controls of matching gender age ranges. The human primary cilia array interrogates expression levels of 84 primary cilia –related genes (Table 2.5). Thermal cycling was performed on a BioRad CFX96 qPCR system according to the manufacturer's instructions. Statistical significance and fold changes in gene expression were calculated after normalisation to all five housekeeping genes associated with the array. Genes of interest were identified on the basis of a fold change of >2 or <-2 and a p value of ≤0.05. Analysis was undertaken using the manufacturer's software accessible on http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php (Qiagen, RT2 profilerPCR array data analysis version 3.5).

Genes of interest were confirmed using gene specific qPCR primers. Primers were designed using primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast). The optimum annealing temperature was determined for each primer set by amplifying cDNA from control URECs and fibroblasts prior to the analysis of experimental samples.

The specificity of each primer set was assessed using bioinformatics software (primer-blast) and verified by assessing melt curves using the Bio-Rad CFX96 qPCR thermal cycler. Quantitative PCR was performed in triplicate and normalised to GAPDH expression using the RT^2 profiler system (Qiagen) according to the manufacturer's instructions.

Table 2.5 Primary Cilia RT2 Profiler PCR Array- list of genes

Mutated in non-motile cilia disease	*AHI1, ALMS1, ARL13B, ARL6, BBS1, BBS2, BBS4, BBS7, CC2D2A, CEP290, GLIS 2, HNF1B (TCF2), INVS, IQCB1, MKKS, MKS1, NEK8, NPHP1, NPHP3, PKD1, PK D2, PKHD1, RPGRIP1L, TMEM67, TTC8*.
Intraflagellar transport	*DYNC2LI1, IFT172, IFT20, IFT74, IFT80, IFT88, KIF3A, KIF3B*
Cilium morphogenesi s	*ALMS1, ARL6, BBS1, BBS2, BBS4, BBS7, IFT172, IFT88, MKKS, OFD1, PKHD1, R PGRIP1L, VANGL2, WWTR1*
Signal transduction	Hedgehog signalling: *BTRC, FUZ, GLI1, GLI2, GLI3, GSK3B, IHH, INTU, LRP2, PTC, H1, RAB23, SHH, SMO, SUFU* cAMP signalling *ADCY3, ADCY7, AVPR2, HTR6, PKD2, SSTR3* mTOR signalling: *AKT1, CDC42, GSK3B, IGF1, INS, MAPK1 (ERK2), MTOR, PIK3CA, PRKCA, RHOA, TP53, TSC1, TSC2* Planar cell polarity (PCP): *DVL1, FAT4, FJX1, FUZ, FZD1, INTU, RHOA, ROCK2, VANGL2, WNT9B.* WNT signalling: *AXIN2, INVS.* PDGFRA/ Integrin signalling: *ITGB1, PDGFRA* *BRAF/MEK/ERK: FOS, KRAS, MAK2K1, MAPK1, MOS, PRKCA, PTPN5*
Cell cycle	*AKT1, BBS4, CCND1, CDK5RAP2, CDKN1A (p21CIP1, WAF1), IGF1, INS, MAP2K1 (MEK1), PKD1, PKD2, TP53*

*List extracted from https://www.qiagen.com/gb/shop/pcr/primer-sets/rt2-profiler-pcr-arrays/?catno=PAHS-127Z#geneglobe

2.13 Anthropometric assessment, fitness assessment and intervention

Anthropometric measurements were undertaken on patients who were in light clothing with shoes removed. Body Mass Index (BMI- weight/height^2, kg/m^2) was converted to BMI-SDS (Body Mass Index Standard Deviation Score) using the British 1990 growth reference data[108,109].

The intervention group participated in once-weekly, individually supervised exercise sessions of 30-60 minute duration, over the course of 28-weeks, which were led by a personal trainer at a local gym facility. An increasing level of intensity of both aerobic and strength training exercises was incorporated to optimise training effect. Personal trainers were asked to track progress and record attendance.

The 10m incremental shuttle walk test (10m-ISWT)[110] was used to assess functional exercise capacity. Total distance covered ('difference in distance' in metres) and level achieved were recorded. Heart rates at rest, maximal exertion and at 3 minutes recovery were monitored using an A300 Polar Watch® (Polar®, Kempele, Finland). Perceived exertion was recorded using the children's OMNI scale [111].

Parents of participants were requested to complete a routine activity diary to record the estimated minutes of activity. Details of activity type, time spent during activity, and number of times per week were recorded. Diaries were completed at baseline and at 28 weeks.

Personal trainers were requested to record the type and duration of exercise for each session. Fitness assessment and management of personal trainers was done by physiotherapists Phillip Harniess and Sean Ledger. Written in collaboration with Dr Sean Ledger and Mr Phillip Harniess.

2.14 MRI Acquisition

Imaging data were acquired using a Siemens Prisma 3T clinical scanner (Siemens Healthcare, Erlangen, Germany) with a 64 channel birdcage head coil. A T1-weighted structural scan was acquired with the following acquisition parameters: TR=2300ms,

TE=2.74ms, TI=909ms and flip angle=9 degrees. In-plane resolution was 1mm, with a 256mm field of view and a slice thickness of 1mm.

The multi-shell diffusion sequence employed a diffusion-weighted spin-echo single shot 2D EPI acquisition, with multi-banded radio frequency pulses to accelerate volume coverage along the slice direction[112,113]. Diffusion-weighted images were acquired along 120 non-collinear gradient directions split over two b-values: 60 directions at b=1000 s/mm^2 and 60 at b=2200 s/mm^2. Spatial resolution was 2.0mm isotropic[114].

Cerebral perfusion was measured using an Arterial Spin Labelling (ASL) sequence, which employed pseudo-continuous labelling (pCASL) in conjunction with a 3D gradient and spin-echo (GRASE) readout. The bolus duration was 1800ms, followed by a post-labelling delay of 1500ms, with background suppression to null the signal from static tissue. In-plane resolution was 3.4 mm, with 24 slices of 4.0mm thickness. Protocol written by Dr Kiran Seunarine.

2.14.1 MR Post-Processing

2.14.1.1 Voxel-based morphometry

For Voxel-Based Morphometry (VBM) analysis images were segmented into Grey Matter (GM), White Matter (WM) and Cerebro-Spinal Fluid (CSF) using the Computational Anatomy Toolbox (CAT12; http://dbm.neuro.uni-jena.de/cat/) for SPM12. A module for processing longitudinal data with an inverse-consistent realignment step and spatial normalisation of the mean image derived from both scans was used, and normalisation into ICBM space. Tissue segments were resampled to a voxel size of 1.5x1.5x1.5mm. Global GM and WM volumes were extracted and summation with CSF volume provided

Total Intracranial Volume (TIV). Images were smoothed using an 8 mm full-width half-maximum Gaussian kernel.

A flexible factorial design with factors group (intervention, control) and time (pre-, and post-intervention) and covariates of no interest (TIV and age) was used to identify the group-wise pattern of longitudinal local GM change[115]. Statistical maps of volume change were displayed using a voxel-wise threshold of $p<0.001$ (uncorrected) with a cluster size of ≥150. Post-hoc voxel-wise regression analyses explored association of local GM volume change with change in physical fitness measures over the follow-up period.

In addition to the VBM analysis, the structural images were also parcellated with Freesurfer to obtain masks of the hippocampi, precentral and postcentral cortex. Protocol written by Professor Torsten Baldeweg.

2.14.1.2 Diffusion Post-Processing

Post-processing of the data was performed using FSL (www.fmrib.ox.ac.uk/fsl) and consisted of distortion correction using 'topup' and 'eddy' followed by brain extraction using Brain Extraction Tool (BET). The diffusion tensor was fit to the corrected multi-shell data using "dtifit" with a weighted-least squares fit, and fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD) and radial diffusivity (RD) maps calculated.

The TBSS (Tract Based Spatial Statistics) pipeline is described in detail in Smith *et al* (2006) [116] and was modified for the purposes of this study. All FA maps (all subjects at both time points) were warped to the FMRIB58 standard space template before averaging the transformed images to create a mean FA map. The mean FA map was then eroded to form an FA skeleton, which was thresholded at 0.2 to remove regions of high

variability. The FA data of each subject was projected onto each voxel of the FA skeleton. Difference maps were generated from the skeletonised FA maps by subtracting the subject's second time point from their first time point. Difference maps allowed for longitudinal analysis of the data. Statistical tests were performed on the difference maps using FSL's randomise tool with 5000 random permutations to ensure convergence. The prior hypothesis for the analysis was that there would be differences in hippocampal microstructure between the intervention and control groups, and that there would be a correlation between microstructural indices and fitness assessment scores. Therefore, multiple comparisons correction was not deemed necessary for this analysis. Protocol written by Dr Kiran Seunarine.

2.14.1.3 ASL Post-processing

Cerebral blood flow (CBF) maps were calculated using the method described by Alsop *et al* (2015)[117]. An affine registration was then used to register each subject's CBF map to their structural T_1 weighted scan, using the 'flirt' algorithm2 in FSL (FMRIB, Oxford, UK).

Hippocampus, pre- and post-central cortex masks, generated using FreeSurfer, where overlaid onto each subject's (T1-registered) CBF map. Median CBF values in each of these regions where calculated, on the left- and right-hand sides of the brain. This was performed for each subject's two imaging sessions, and the change in the median CBF value (ΔCBF) between the two time points was recorded in each region. Linear regression was used to determine the relationship between ΔCBF values in each region (dependent variable), and measured values of 'exercise minutes gained', and 'difference in distance' (independent variables). Protocol written by Dr Patrick Hales.

2.15 Intelligence quotient testing

Intellectual ability was assessed with the Wechsler Abbreviated Scale of Intelligence – Fourth Edition (WASI-IV). Two subtests were administered: Vocabulary and Matrix reasoning, providing measures of verbal and performance IQ, respectively.

2.16 Memory testing ('Pair Games')

Memory was assessed using an experimental protocol (the "Pair Games")[118] which assesses learning and memory through recall. The Pair Games is a paired-associated learning paradigm, which consists of learning pairs of items over three consecutive trials administered via a tablet.

The participants were presented with 10 pairs of abstract designs. Subsequently they were presented with the first item of each pair and were asked to recall by drawing the paired item. This was repeated three times in a row to provide a measure of learning. After a 15-minute delay, the participants were asked again to recall as many pairs as possible; this procedure provides a measure of delayed recall. The score of learning is calculated by average scores across learning trial, and the score of forgetting is calculated by subtracting the score at the delayed trial from the score obtained at the third trial. This reflects the amount of learnt items that are forgotten after a delay and provides a measure of retention. A score of 0 indicates forgetting of information after a delay.

Two parallel versions of the paradigm were used for the assessment of learning and memory before and after exercise intervention. Written in collaboration with Dr Sarah Buck.

2.17 Statistical analysis

2.17.1 Statistical analysis -'Renal phenotype and natural history study' (Chapter 3)

Genotype-phenotype analysis was targeted to patients with mutations in the most commonly affected genes *BBS1* and *BBS10* but also included *BBS2*, *BBS9* and *BBS12* where possible. Mutation type- phenotype analysis was also assessed. Patients with two missense mutations were compared with two truncating (nonsense or frameshift) mutations and a combination of missense/truncating mutations. Children were not included in genotype and mutation type analysis of end-stage renal failure since chronic kidney disease staging only becomes relatively stable in adulthood. Multivariable regression analysis was used to evaluate the effect of confounders as well as genotype-phenotype analysis. In the multivariable regression analysis the odds ratio describes the relative burden of each risk factor. The Mann–Whitney U test was performed to assess differences in median age for genotypes *BBS1* and *BBS10*. Statistical analyses were conducted in R (R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from: http://www.R-project.org/). The code for R was written by Dr Manolis Bagkeris (Statistical Support Service, UCL Institute of Child Health). A 5% confidence level was considered statistically significant. All tests were two tailed.

2.17.2 Statistical analysis –'Eye phenotype and natural history study' (Chapter 4)

Genotype-phenotype analysis was targeted to patients with mutations in the three most commonly affected genes *BBS1, BBS2* and *BBS10*. Correlation with mutation type was also assessed. Patients with two missense mutations were compared to patients with either two truncating mutations or missense/truncating mutations. Statistical analyses were conducted in R (R Core Team (2013). R: A language and environment for statistical

computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from: http://www.R-project.org/). A 5% confidence level was considered statistically significant. All tests were two tailed.

2.17.3 Statistical analysis -'Obesity phenotype and natural history study' (Chapter 5)

Genotype-phenotype analysis was targeted to patients with mutations in the two most commonly affected genes *BBS1* and *BBS10*. Correlation with mutation type was also assessed. Patients with two missense mutations were compared to patients with either two truncating mutations or missense/truncating mutations. Statistical analyses were conducted in R (R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from: http://www.R-project.org/). The paired T test was used to compare paired changes in BMI/ BMI-SDS. A 5% confidence level was considered statistically significant. All tests were two-tailed.

2.17.4 Statistical analysis –'Cardiovascular phenotype and natural history study' (chapter 6)

Genotype-phenotype analysis was targeted to patients with mutations in the two most commonly affected genes *BBS1* and *BBS10*. Correlation with mutation type was also assessed. Patients with two missense mutations were compared to patients with either two truncating mutations or missense/ truncating mutations. The non-parametric Mann-Whitney U test, Kruskal-Wallis test and ANOVA were performed as appropriate to identify associations between genes (*BBS1* versus *BBS10*) and mutation types (two truncating mutations, heterozygous missense/ truncating mutations or two missense mutations).

Multivariable regression analysis was applied to variables which were statistically significant on univariable analysis and/or known indicators of cardiometabolic disease. A 5% confidence level was considered statistically significant. All tests were two tailed. Statistical analyses were carried out using SPSS version 21.0 (SPSS, Inc., Chicago IL) in collaboration with Dr Manolis Bagkeris (Statistical Support department, UCL Great Ormond Street Institute of Child Health). Multivariable regression analysis was performed without statistical correction.

2.17.5 Statistical analysis –'Cellular phenotype study' (chapter 7)

Statistical analysis in this chapter was completed using GraphPad Prism software version 6.01 (GraphPad Software Inc., La Jolla, CA, USA). All data are shown as the mean ± standard error. Comparison of two groups was done through the Mann-Whitney U test. A *p* value of <0.05 was considered statistically significant.

2.17.6 Statistical analysis for the study examining the effect of exercise on the hippocampus (Chapter 8)

Statistical analysis of physical activity assessments was completed using IBM® SPSS® Statistics 24 (Chicago, IL, USA). All fitness results were expressed as mean ± standard deviation (SD), and 95% confidence intervals (95%CI). Comparison of groups was done using the non-parametric Mann-Whitney-U test. A p value of ≤0.05 was considered statistically significant.

Statistical analysis for the cognition and memory element of this study was conducted using IBM® SPSS® Statistics 24 (Chicago, IL, USA). All results were expressed as mean ±SD and 95% confidence intervals (95%CI). Comparison of groups was done using the non-

parametric Mann-Whitney-U test. A p value of ≤0.05 was considered statistically significant.

Statistical methods applied to imaging results are described in detail in conjunction with the imaging study protocols (section 2.12).

3 Renal disease in Bardet-Biedl syndrome: characterising the phenotype and predicting the outcome

"Will my child develop kidney disease? If so when? Could they die from it? Can you treat it?"

3.1 Introduction

Renal dysplasia and functional deficits are a significant cause of morbidity and mortality in BBS that are reported to affect 53-82% of patients[3,56,119-122]. Bladder and other urinary tract dysfunction is frequently described by patients, but poorly documented in the literature[120]. Primary renal disease in Bardet-Biedl syndrome is typically described as polycystic disease[3], but dysplastic disease, focal sclerosing glomerular disease and concentrating defects have also been described[56,119,121-123]. Renal disease may also occur as a consequence of metabolic disease, hypertension and diabetes, all of which are prevalent in the BBS population[4]. Renal disease is a cause of significant anxiety for many patients with Bardet-Biedl syndrome due to the effect on quality of life, morbidity and mortality[119,121,123].

3.1.1 Molecular pathogenesis of renal disease in Bardet-Biedl syndrome

The primary cilium is thought to play a key role in nephrogenesis[124]. This hypothesis is corroborated by the high prevalence of renal disease in ciliopathies including PKD, SLS, JATD, Nephronophthisis and others[2]. A characteristic renal finding amongst all these conditions is the presence of cysts.

Primary cilia are expressed throughout the kidney including in the nephron, collecting ducts and parietal layer of Bowman's capsule[125]. Evidence from rats suggests that glomerular podocytes express cilia during embryogenesis but these are usually not present in adulthood[126].

The primary cilium serves as a mechanoreceptor in the kidney deflecting in response to the flow of fluid[127]. This results in activation of a calcium channel and intracellular cilia-associated calcium signalling is subsequently increased[128]. It has been hypothesised that defective intracellular calcium signalling contributes to cell proliferation and cyst formation in renal ciliopathies[128].

The molecular mechanisms underpinning the onset of cystic kidney disease in the ciliopathies is not well understood. However, studies on Autosomal Dominant Polycystic Kidney Disease (ADPKD), the commonest ciliopathy, provide a model for the aberrations in cilia signalling that lead to cystic disease, which are likely to share some common features with the development of cystic kidney disease in other ciliopathies including BBS.

The loss of function of either of the ADPKD associated polycystin proteins (PC1 and PC2) is thought to lead to the developing of renal cystic disease through aberrant signalling in pathways including planar cell polarity (PCP), Wnt, mammalian Target Of Rapamycin (mTOR), cyclic Adenose MonoPhosphate (cAMP), G Protein Coupled Receptor (GPCR), Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), Epidermal Growth Factor Receptor (EGFR), Mitogen Activated Protein Kinase (MAPK), cellular calcium and the cell cycle[129]. PC1 and PC2 form a complex that helps maintain intracellular calcium homeostasis[130]. Disruption of either PC1 or PC2 is thought to lead to decreased intracellular calcium, upregulated cAMP and increased cell proliferation[131]. Excessive renal tubular epithelial cell proliferation and remodelling of the surrounding tissue leads to the development of cysts and fibrosis, and destruction of the normal renal parenchyma, resulting in enlarged kidneys and deterioration in renal function[128].

Evidence from studies in BBS mouse models is supportive of similar mechanisms of pathogenesis in BBS. Mouse models of BBS including *Bbs2-/-*[132] and *Bbs4-/-*[133] both develop kidney cysts in the presence of structurally normal primary cilia, consistent with the findings in animal models of ADPKD[134]

Tobin *et al* demonstrated that Rapamycin and Roscovitine could reverse renal aberrations in three zebrafish models of Bardet-Biedl syndrome (*bbs4*, *bbs6*, *bbs8*) suggesting a possible role for mTOR signalling on BBS renal dysplasia[59]. BBS proteins are also known to play an essential part in PCP[135] and Wnt signalling[136], both of which are implicated in ADPKD related cystogenesis.

3.1.2 Classification of chronic kidney disease in BBS

Chronic kidney disease (CKD) in BBS has hitherto been treated empirically in clinic, and in line with other causes of CKD due to a lack of clinical evidence and disease- specific guidelines.

The 'Kidney Disease: Improving Clinical Outcomes (KDIGO)' report on chronic kidney disease was first published in 2002[137] and described the best practice for evaluating, classifying and managing chronic kidney disease in the general population. Controversial in parts, guidelines developed as a result of the KDIGO report have been credited with reducing the number of people who are referred late for renal transplants or renal replacement therapy[138]. The guidelines have, however, also been criticised for poor correlation with adverse outcomes including all-cause mortality, end stage renal disease and cardiovascular risk[139,140]. They rely entirely on estimated Glomerular Filtration Rate (eGFR) as a biomarker of renal function and divide chronic kidney disease into five stages with associated predicted renal outcomes (Table 3.1). An eGFR<45 ml/min per 1.73 m2

correlates to CKD3b-5 and is generally accepted as representing severe chronic kidney disease.

Table 3.1 Chronic Kidney Disease classification

Chronic Kidney Disease stage 1	eGFR>90 ml/min per 1.73 m^2. Normal function. Structural abnormalities only.
Chronic Kidney Disease stage 2	eGFR: 60-89 ml/min per 1.73 m^2. Mild reduction in renal function.
Chronic Kidney Disease stage 3	eGFR: 30-59 ml/min per 1.73 m^2. Significant reduction in renal function.
Chronic Kidney Disease stage 4	eGFR:15-29 ml/min per 1.73 m^2. Severe reduction in renal function.
Chronic Kidney Disease stage 5	eGFR<15 ml/min per 1.73 m^2. Renal failure.

Table adapted from K/ DOQI[137]

Modified guidelines have been developed[141] to include albumin-creatinine ratio as both eGFR and albumin-creatinine ratio have been shown to have be independent risk predictors of morbidity and mortality[141]. Albumin-creatinine ratio is a proxy for microvascular circulation and a validated risk predictor of adverse cardiovascular outcome[142]. New guidelines produced by the National Institute for Clinical Excellence (NICE), which also factor in other diagnoses such as diabetic nephropathy, have thus far not been widely implemented into clinical practice[138].

Estimated GFR and albumin-creatinine ratios are routinely measured and co-morbidities registered in the UK national BBS clinics, but no disease-specific guidelines have previously been developed.

3.1.3 Rationale

There is significant variability in the BBS renal phenotype. Before this study was completed patients and clinicians were unable to predict which patients were likely to develop renal disease. Very little was understood about the likelihood of developing end stage renal disease, which has a significant effect on quality and life and potentially mortality. Furthermore, understanding the variation in BBS kidney structure and function as well as the natural history of BBS renal disease provides the basis for future therapeutic development.

3.2 Results

Patients participating in this study attended the UK national Bardet-Biedl syndrome clinics in either Birmingham or London during a four year period (2010-2014). The age range of patients attending the BBS clinics varied from newborn to 60 years old. The peak age at first attendance was 6-10 years old, and relatively few older adults attended the clinics (figure 3.1).

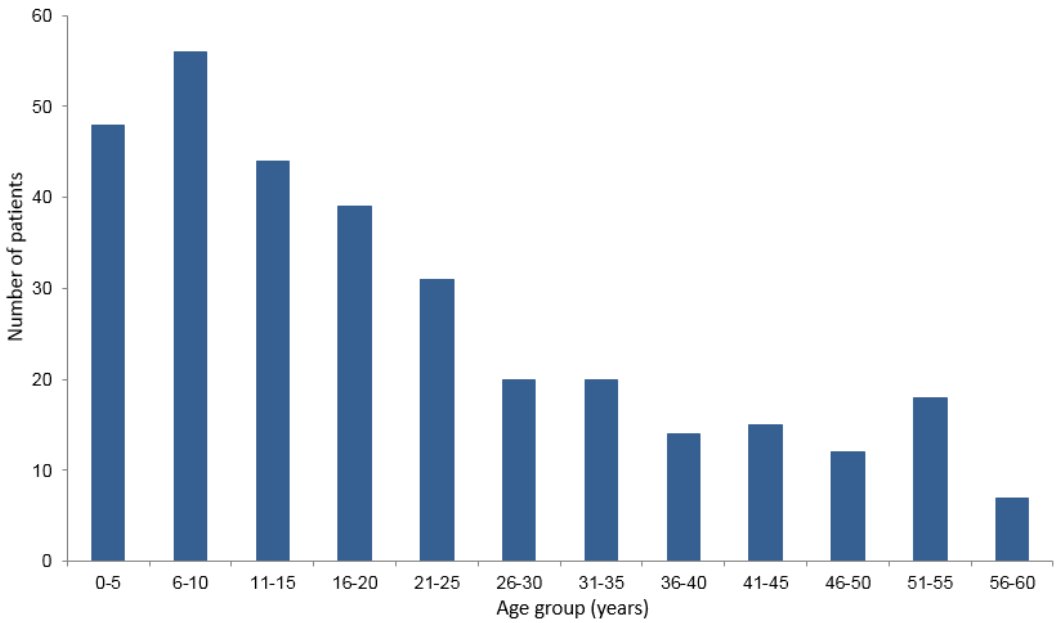


Figure 3.1 Age at presentation to the UK national BBS clinics
Note the peak in the 6-10 year age group reflecting the age patients are typically diagnosed with rod-cone dystrophy. No patients older than 60 attended the clinics at the time this study was conducted, most likely representing an ascertainment bias. Adapted from Forsythe *et al,* 2017[1]

Patients were included in the study if they had a clinical diagnosis of BBS. All patients had genetic testing and molecular confirmation of the diagnosis was achieved in 77% of all patients (265 out of 350 patients). Fifty four percent of responders were male and 46% were female. Figure 3.2 demonstrates the distribution of genotypes. In order to facilitate statistical analysis mutation types were analysed according to likely severity. One hundred and twenty five patients had two missense mutations, 82 had two truncating mutations (frameshift, nonsense, splice site), 39 had a combination of missense and truncating mutations and the remaining fraction of patients had other mutations types of uncertain severity including exon deletions and start codon mutations.

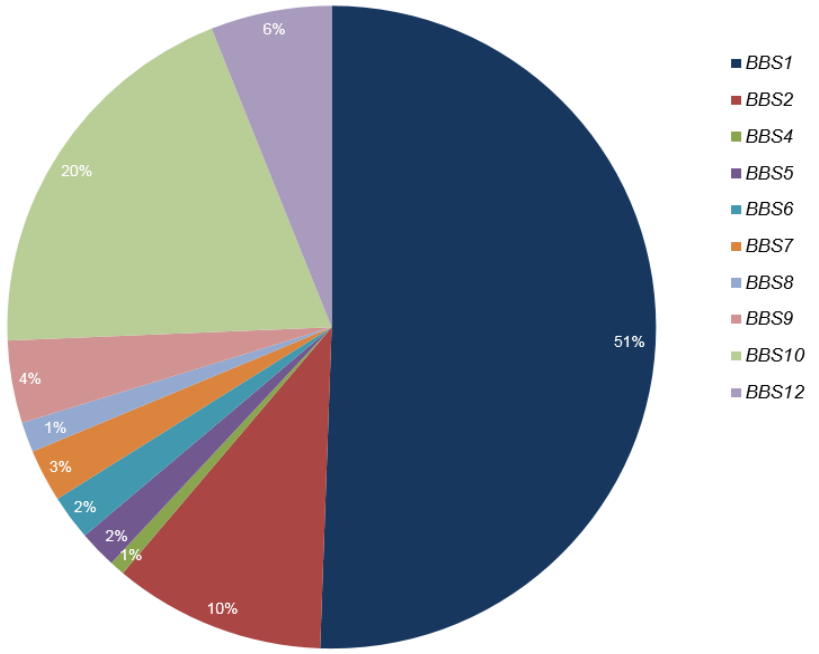


Figure 3.2 Distribution of genotypes in this study
Note the high percentage (51%) of patients with mutations in *BBS1*. Mutations in *BBS10*, BBS2 and *BBS12* are also frequent (20%, 10% and 6% respectively). Adapted from Forsythe *et al,* 2017[1]

3.2.1 Primary renal disease in BBS presents in early childhood

Medical records for all patients attending the paediatric clinics in London and Birmingham were reviewed, assessing the earliest recorded age of onset of chronic kidney disease stages 2-5 (CKD2-5). Forty nine patients of 149 (32%) paediatric clinic attenders had chronic renal disease. Figure 3.3 demonstrates the earliest age at presentation as well as the CKD stage. All patients with end stage kidney disease (CKD4-5) were diagnosed in early childhood (aged 0-4 years old). Patients presenting with milder forms of renal disease presented throughout childhood. This may reflect an ascertainment bias; the peak age at presentation to the BBS clinic is 6-10 years old (figure 3.1), usually prompted by the onset of eye disease. It is usually following the BBS diagnosis that patients have their first renal sonography and renal function tests and renal impairment is discovered.

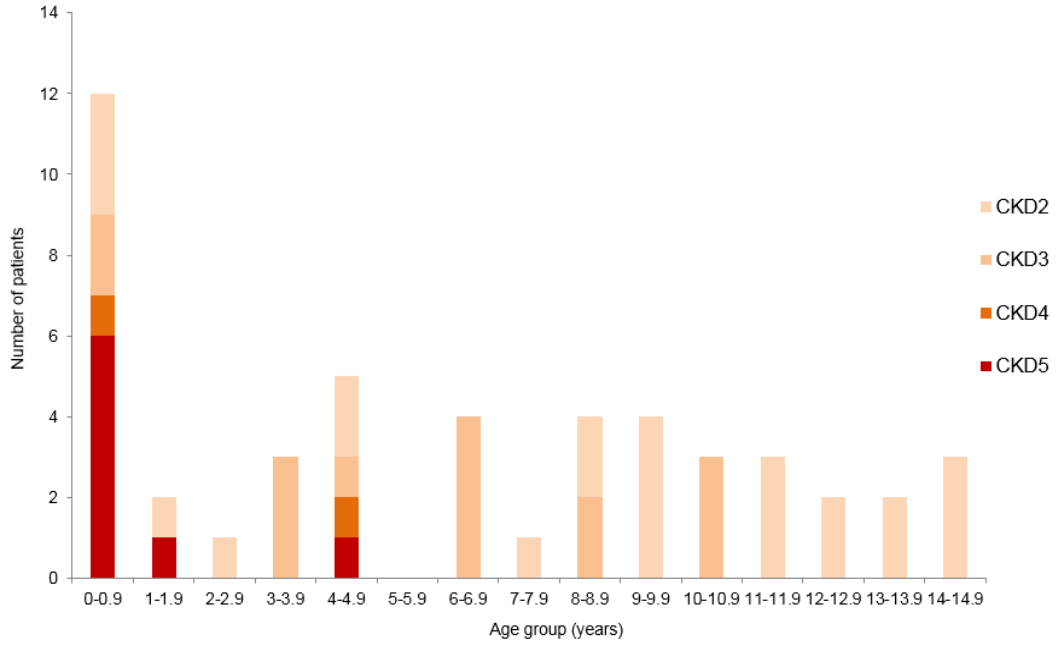


Figure 3.3 Age and stage of CKD at first recorded presentation with renal disease

Severe renal disease usually presents in the first 4 years of life. CKD2-3 may present at any age. Adapted from Forsythe *et al,* 2017[1]

The age of onset of CKD5 was ascertained retrospectively and charted in a Kaplan-Meier survival curve (figure 3.4) demonstrating that the majority of patients (14; 70%) reach CKD5 by the age of 20.

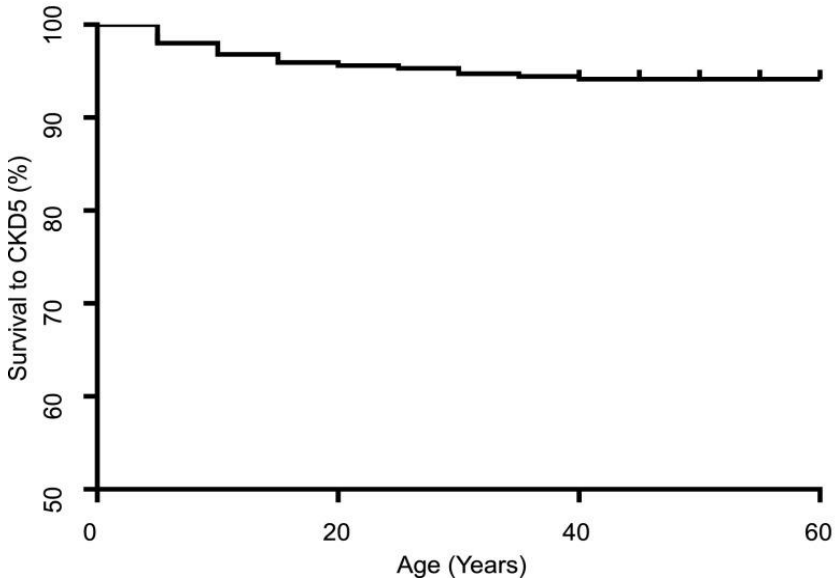


Figure 3.4 Kaplan-Meier survival demonstrating that the majority of patients with CKD5 (n=14; 70%) have reached end-stage kidney disease by age 14
Adapted from Forsythe *et al,* 2017[1]

3.2.2 Renal disease is less prevalent and less frequently severe than previously reported

Assessment of the prevalence and CKD stage of kidney disease in the paediatric and adult populations (Figure 3.4, Table 3.1) revealed several surprising observations. Firstly, the prevalence of kidney disease was lower than expected in that 69% of children and 58% of adults had no known functional renal deficit. Furthermore, only a small fraction of children and adults had end-stage renal disease (6% and 8% respectively). This low prevalence is a positive finding, and the small difference in end-stage kidney disease between children and adults indicates that relatively few individuals who do not have severe kidney disease in childhood go on to develop end stage kidney disease in adulthood. This is consistent with previous anectodal observations from the UK national BBS clinics which suggest that patients who develop severe primary kidney disease do

so in early childhood, and that most other patients maintain normal or near- normal kidney function as opposed to the slow decline observed in the most common renal ciliopathy polycystic kidney disease (PKD)[143]. Table 3.2 summarises how CKD stage was assessed in the paediatric and adult populations.

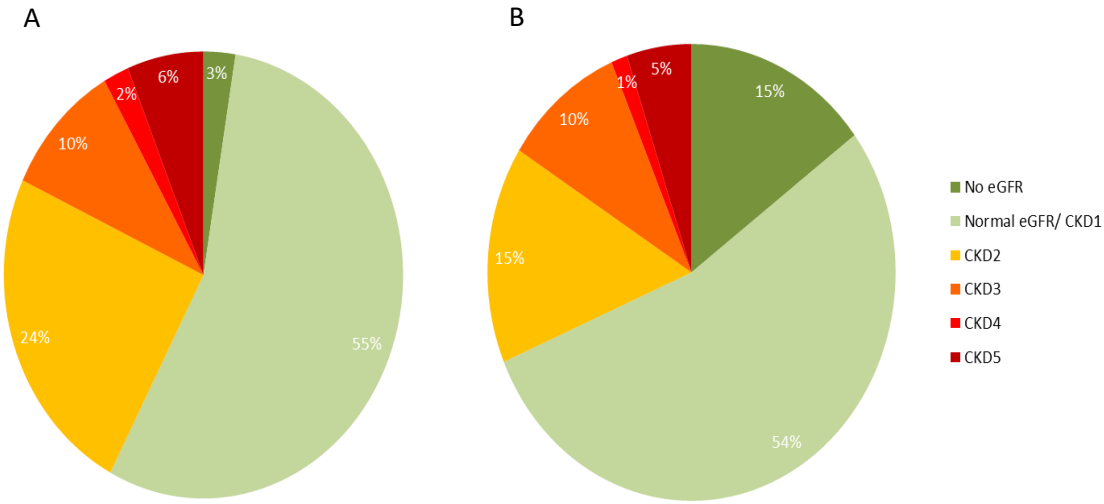


Figure 3.5 Percentage distribution of CKD stages 2-5 based on eGFR results in (a) adults (n=194) and (b) children (n=156)

Less than half of patients develop renal function impairment, and relatively few patients have end stage renal disease. Adapted from Forsythe *et al,* 2017[1]

Table 3.2 Assessment for CKD.

CKD Marker	**n/Total (% of Total)**	
	Paediatric Patients	*Adult Patients*
Total no. of patients	156 (100%)	194 (100%)
eGFR		
Total no. of patients who had eGFR	133/156 (86%)	189/194 (97%)
Normal or CKD1	84/133 (63%)	107/189 (57%)
CKD2–5 (<90 ml/min per 1.73 m2)	49/133 (37%)	82/189 (43%)
CKD5 (<15 ml/min per 1.73 m2)	8/133 (5%)	12/189 (8%)
Renal ultrasound scan	87/156 (55%)	90/194 (46%)
Normal	44/87 (51%)	43/90 (48%)
Abnormal	43/87 (49%)	47/90 (52%)
No ultrasound scan and no eGFR	9/156 (6%)	5/194 (3%)
Urinalysis	140/156 (90%)	186/194 (96%)

Adapted from Forsythe *et al,* 2017[1]

Table 3.3 Prevalence of CKD in adults and children

Age group/ years in age	**Normal/CKD1/No eGFR**	**CKD2**	**CKD3**	**CKD4**	**CKD5**	**Total**
Pediatric patients, n						
0–5	22	2	7	0	5	36
6–10	42	7	5	2	1	57
11–15	29	6	2	0	1	38
16–18	14	9	1	0	1	25
Total (% of total)	107 (69%)	24 (15%)	15 (10%)	2 (1%)	8 (5%)	156
Adult patients, n						
16–20	27	0	3	0	2	32
21–25	29	5	1	1	3	39
26–30	17	5	1	1	1	25
31–35	14	4	1	1	3	23
36–40	6	7	5	0	1	19
41–45	5	8	2	1	1	17
46–50	6	5	2	0	1	14
51–55	6	8	3	0	0	17
56–60+	2	5	1	0	0	8
Total (% of total)	112 (58%)	47 (24%)	19 (10%)	4 (2%)	12 (6%)	194

Adapted from Forsythe *et al,* 2017[1]

3.2.3 Urinary albumin-creatinine ratio correlates with severe renal disease in BBS

Urinary albumin-creatinine ratio was used as a proxy for glomerular injury. One hundred and thirty nine patients had urinary albumin/ creatinine measurements, and of these 119 could be matched to eGFR. Five percent (n=7) had macroalbuminuria (albumin-to-creatinine ratio >30 mg/mmol) three of whom were diabetic, 28% had microalbuminuria (albumin-to-creatinine ratio >3.5 mg/mmol) two of whom were diabetic. Correlation with eGFR was statistically significant at p=0.01.

3.2.4 There is significant variability in the spectrum of structural renal disease in BBS

In order to assess the spectrum of structural renal disease in BBS, renal ultrasound reports from 177 patients were reviewed. Eighty seven (49%) reports did not reveal any abnormalities, the remaining 90 reports revealed a selection of structural abnormalities. These were divided broadly into six categories: polycystic/ dysplastic disease, hydronephrosis, atrophic/scarring, echogenic/ loss of corticomedullary differentiation or developmental abnormalities (figure 3.6). Significant diversity in the structural defects was evident ranging from single to multiple cysts and horseshoe, duplex, ectopic or absent kidneys.

Structural renal abnormalities correlated statistically with the presence of severe renal disease (p=0.04); all patients with severe renal disease (CKD3b-5) who had a renal sonography report available had a structural abnormality present (n=7).

Structural abnormalities were not always detected antenatally despite both antenatal and postnatal sonography being completed. Thirty nine paediatric patients with known renal structural abnormalities had both antental and postnatal sonography. Of these, 14 patients (36%) failed to be diagnosed antenatally.

Thirty patients presented with cystic kidney disease which is classically associated with BBS. Of these patients, 24 had molecular confirmation of BBS- primarily *BBS1* or *BBS10*.

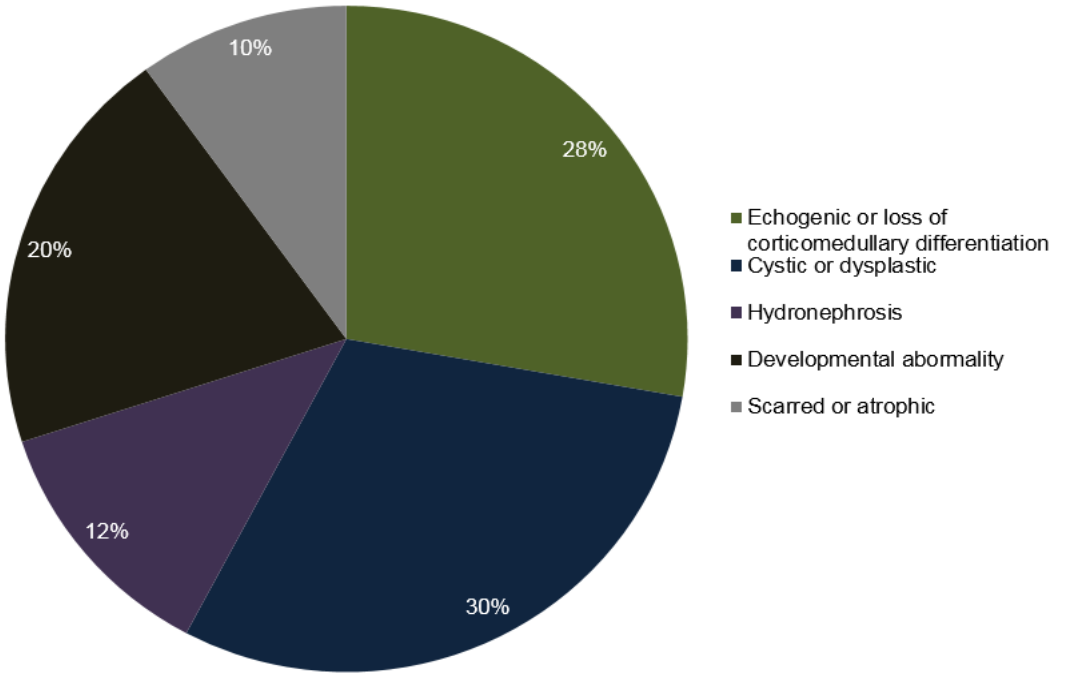


Figure 3.6 Prevalence of renal structural abnormalities present on renal ultrasonography (n=90).
Of note, only 30% have evidence of cystic/ dysplastic disease characteristic of renal disease in the ciliopathies. Adapted from Forsythe *et al,* 2017[1]

3.2.5 Patients with BBS are at high risk of secondary renal disease due to hypertension and diabetes

Sixty seven (35%) of all adults participating in this study were hypertensive. The presence of hypertension correlated statistically with CKD3b-5 and with patients on antihypertensive medication (p=0.01). There was also a significant correlation between hypertension and albuminuria (p<0.001).

Twenty eight (15%) of all adults were on hypoglycaemic medication. There was no statistically significant correlation with CKD3b-5 (p=0.47).

3.2.6 Genotype and mutation type predict likelihood of developing severe kidney disease

On univariable statistical assessment of genotype-phenotype correlations in adults with BBS a significant correlation was identified between the presence of severe renal disease (CKD3b-CKD5) and genotype. Patients with mutations in *BBS2*, *BBS10* and *BBS12* were more likely to have severe kidney disease than patients with mutations in *BBS1* ($p= 0.02$, $p<0.001$, $p=0.03$ respectively). Similarly, univariable analysis of mutation type – phenotype correlations revealed that patients with truncating mutations or truncating/ missense mutations were more likely to severe renal disease compared to patients with two missense mutations ($p<0.001$, $p=0.01$) (table 3.4).

On comparison between patients with the two most common genotypes *BBS1* and *BBS10* of the relative frequency of different stages of CKD, patients with *BBS1* mutations were more likely to be disease free or have early stage CKD (figure 3.7a). The higher incidence of early stage CKD should be considered in the context of the patient population where patients with *BBS1* mutations were statistically older than patients with *BBS10* mutations ($p=0.001$) and therefore more likely to have renal disease secondary to metabolic syndrome, diabetes or hypertension.

Given the larger sample size assessed for this study an attempt was made to assess if patients with the recurrent *BBS1* p.Met390Arg missense mutation demonstrated a hypomorphic renal phenotype commensurate with the clinical impression and previous research finding of an overall milder phenotype[4,144]. On comparison of patients who are homozygous for *BBS1* p.Met390Arg with other *BBS1* mutation combinations no hypomorphic effect was evident (figure 3.7b). As with other genotype-phenotype assessments presented here, it was not possible to compare homozygous *BBS1* p.Met390Arg patient data to data from patients who did not have at least one

p.Met390Arg mutation due to very small number, since most patients with mutations in *BBS1* are at least heterozygous for p.Met390Arg (figure 3.7b). Due to the autosomal recessive inheritance pattern of BBS, the presence of just one hypomorphic allele may compensate for a more severe allele hence ameliorating the phenotype. However, it is significant that out of 95 patients who were homozygous for *BBS1* p.Met390Arg only one patient has had end stage renal disease.

Due to small numbers it was not possible to assess for genotype-phenotype correlations underlying patients' comparative urinary albumin-creatinine ratios. There was no genotype-phenotype correlation found between the presence or absence of structural defects (*BBS1* versus *BBS10*, p=0.19).

Table 3.4 Univariable logistical regression analysis of risk factors for severe renal disease (eGFR<45 ml/min per 1.73 m2) in adults with the most common genotypes

Risk Factor	**Odds Ratio**	**Confidence Interval**		**P Value**
		2.5%	97.5%	
Genetic factors				
Genotype (n=154)				
BBS1 mutation (n=90)	(Reference)			
BBS2 mutation (n=22)	4.4	1.28	15.19	0.02*
BBS9 mutation (n=6)	2.4	0.12	17.74	0.46
BBS10 mutation (n=26)	7.4	2.49	23.32	<0.01*
BBS12 mutation (n=10)	5.9	1.08	28.39	0.03a
Mutation type (n=149)				
Missense/missense (n=76)	(Reference)			
Truncating/truncating (n=40)	11.4	3.9	41.8	<0.01*
Missense/truncating (n=33)	6.3	1.5	28.6	0.01*
Non-genetic factors				
Diabetes (n=137)	0.62	0.14	0.99	0.47
Hypertension (n=137)	5.43	2.21	14.29	<0.01*
Body mass index (n=93)	1.04	0.96	1.10	0.32
Age (n=154)	1.02	0.99	1.96	0.15

*Statistically significant result.
Adapted from Forsythe *et al,* 2017[1]

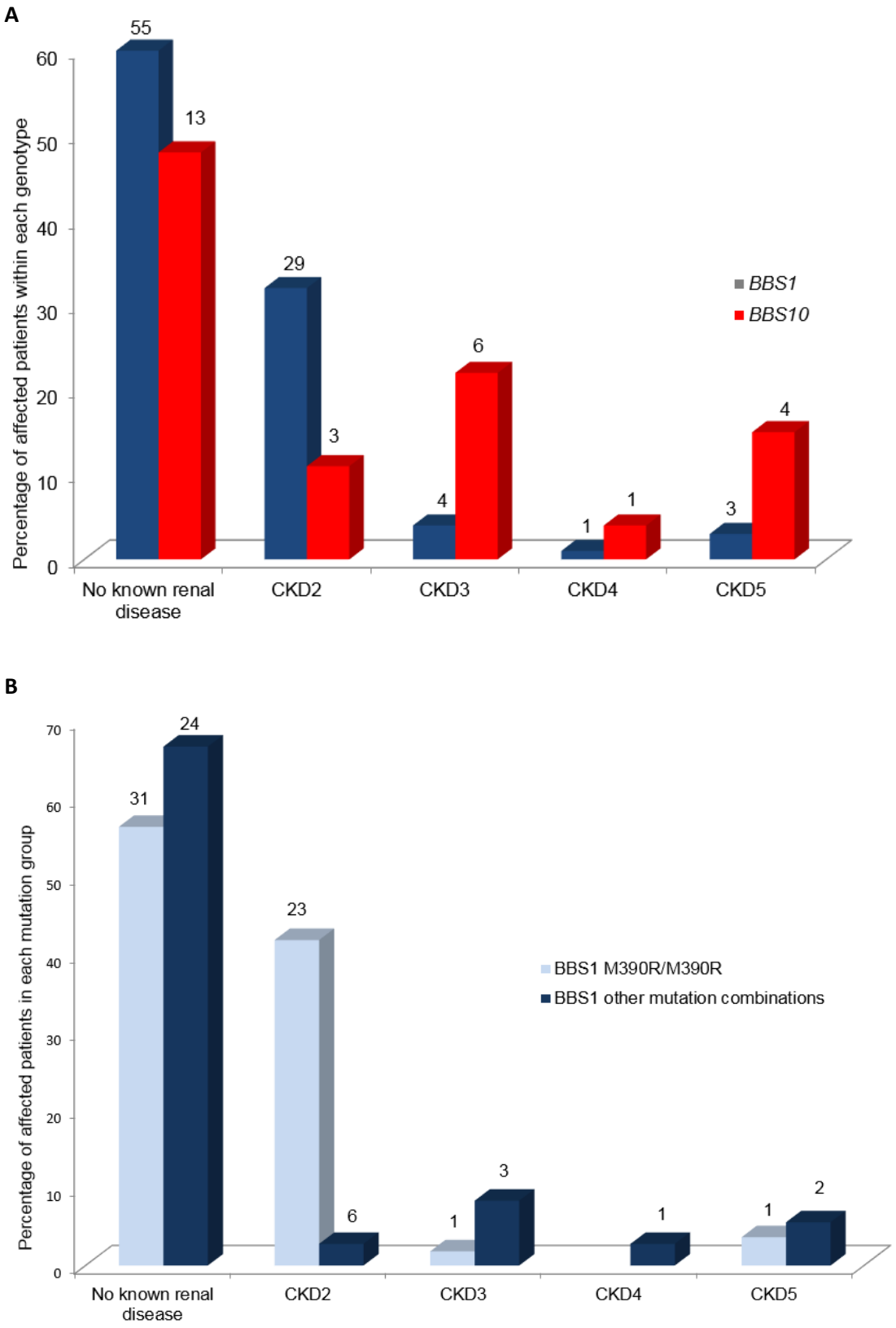


Figure 3.7 Distribution of CKD stages in adults with different genotypes/ mutation types

A. Percentage distribution of CKD stages in adults. Mutations in BBS1 (n=92) versus BBS10 (n=27). B. Subanalysis of adults with *BBS1* mutations only. Percentage distribution of CKD stages in *BBS1* p.Met390Arg/p.Met390Arg (n=55) versus other *BBS1* mutation types (n=36). Adapted from Forsythe *et al.* 2017[1]

3.2.7 Clinical management of renal disease in BBS can be structured according to risk

This study has identified a number of risk factors for developing severe renal disease in BBS, including genotype, mutation type, sonographic evidence of structural abnormalities, proteinuria, hypertension and diabetes, and a relative protective factor in the form of reaching adulthood without developing renal disease. These factors are incorporated into a risk factor-dependent tiered paediatric and adult management approach as outlined in figure 3.8. This management approach would entail that adults with the lowest risk of renal disease could receive community nephrology follow up and low risk children could be seen less frequently in specialist clinics. All patients with end stage renal disease require frequent specialist follow up and those with identifiable risk factors or early, stable renal disease (CKD1-3) warrant annual specialist follow up.

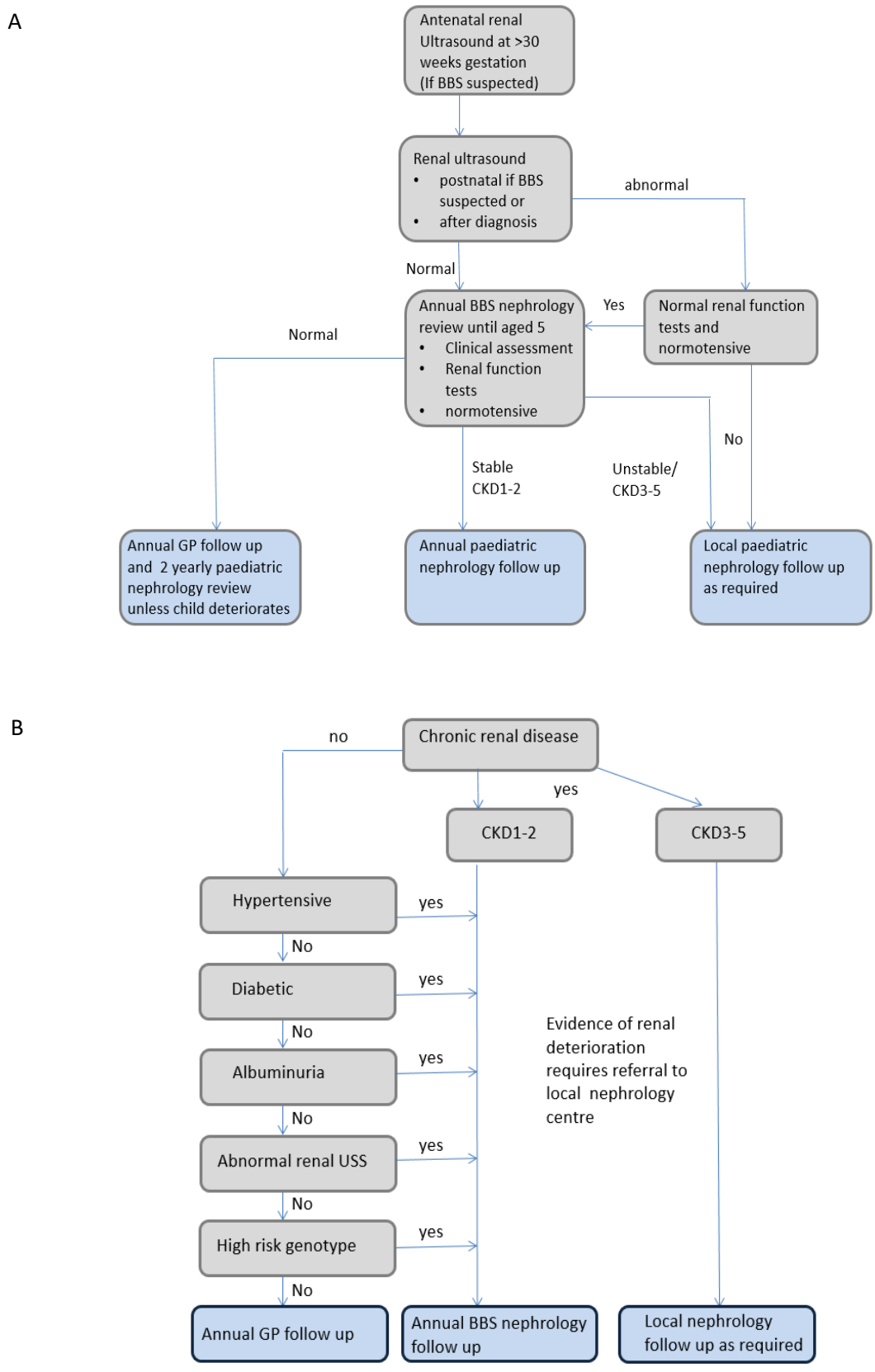


Figure 3.8 Proposed clinical management of renal disease in BBS

A. Proposed paediatric management pathway. B. Proposed adult management pathway. Patients are stratified according to risk factors for developing severe renal disease and nephrology follow up is organised accordingly. Patients with end stage renal disease are seen locally as clinical input needs supersede management capacity in the BBS clinics.

3.3 Discussion

This is the largest reported study to date examining the renal phenotype in BBS. The variation of genotypes in this study reflects the UK BBS population. Compared to the previously reported prevalence[6], *BBS1* is relatively overrepresented. This may reflect the high prevalence of Caucasian patients and is reflective of the UK genotype population. It is notable that patients with *BBS1* mutations were significantly older than patients with mutations in *BBS10*. This may be due to the mean later onset of visual loss in patients with *BBS1* mutations.

CKD is less prevalent in this cohort of BBS patients than expected based on previous reports[56]. Thirty one percent of children and 42% of adults have CKD2-5 and only 6% and 8% respectively develop CKD4-5.

There was a statistically significant correlation between the presence of structural renal aberrations and CKD in this cohort although not all patients with structural aberrations go on to develop a functional deficit. Evidence from this study suggests that every patient should have a baseline renal ultrasound examination to assess for the presence of structural abnormalities[6]. If a structural aberration is identified close monitoring is required.

Although mutations in *BBS10* have previously been reported to have a higher incidence of antenatal severe polycystic kidney disease[145], there was no evidence in this population that the rate of pregnancy losses was higher in families with children who had *BBS10* mutations, and the prevalence of sonographic evidence of cystic kidney disease in this *BBS10* population was consistent with the overall BBS population (21% versus 20%).

A previous study suggested that proteinuria is not present in BBS renal disease[146]. This study disputes this finding. Furthermore, I was able to demonstrate that there is a

statistically significant correlation between CKD3b-5 and albuminuria supporting the revised CKD NICE guidelines including albumin-creatinine ratio in the risk assessment of CKD patients.

This study revealed statistically significant genotype-phenotype and mutation type – phenotype correlations. Patients with *BBS1* mutations and missense mutations were significantly less likely to develop severe CKD. The *BBS1* missense mutation hypomorphic phenotype is consistent with the milder spectrum of findings identified in the visual function of patients with BBS (as described in chapter 4), as well as the milder cardiovascular phenotype (chapter 5). Ascertaining if this effect relates to a hypomorphic effect of the *BBS1* p.Met390Arg missense variant or whether *BBS1* and missense mutations in other BBS genes exert independent hypomorphic effects could not be elucidated. However, it is noteworthy that only one of 90 patients with *BBS1* p.Met390Arg mutations developed CKD4-5. The higher prevalence of *BBS1* p.Met390Arg mutations in this population compared to BBS patient groups previously reported by other investigators could also account for the lower prevalence of renal disease in our cohort.

Increasing demands and financial constraints in the NHS require frequent re-assessment and optimisation of clinical care including for patients with rare diseases. Patient stratification according to the risk factors identified in this study can optimise patient care for BBS patients. For those patients at low risk of renal disease this could lead to fewer hospital visits and less frequent disruption to school or work attendance. Previous recommendations advise that patients should be reviewed by a nephrologist annually unless renal failure is present in which case closer monitoring is required. Based on the proposed management pathway in figure 3.8 adults with the lowest risk of renal disease could receive community nephrology follow up and low risk children could be seen less

frequently in specialist clinics. All patients with end stage renal disease require frequent specialist follow up, and those with identifiable risk factors or early stable renal disease (CKD1-3) warrant annual specialist follow up. A multi-national study could facilitate the development of a statistical renal risk calculator for this unique population.

Limitations The age distribution assessed in this study is most likely a reflection of ascertainment bias rather than reflecting the true age distribution of people living with BBS in the UK. The peak in the 6-10 year age range most likely reflects the fact that this is the peak age for children to be referred to the service since this is when children are most commonly diagnosed with rod-cone dystrophy. The small number of older adults in the clinics is likely a reflection of older adults not receiving a diagnosis rather than an actual increase in the number of people with BBS over time.

Not all patients had a full renal assessment including renal function tests, urinary albumin-creatinine ratio and renal sonography and it is therefore possible that some of the patients who remain without renal function tests have chronic kidney disease.

I discuss the high prevalence of antihypertensive medication in BBS patients in chapter 5. This study was not able to discern if this was prescribed as part of CKD management or for idiopathic hypertension as the two often coincide in the same patient.

Future direction This study has informed the clinical management of kidney disease in the UK national BBS clinics. There is now evidence attesting to the lower prevalence of kidney disease than previously reported, the relatively low incidence of end-stage kidney disease, and –in those who develop primary kidney disease- early age of onset. A multi-national study could facilitate a better understanding of the whether *BBS1* and missense mutations exert independent hypomorphic effects and enable the development of a statistical renal risk calculator for this unique population.

This is the biggest published study to date[1] characterising kidney disease in BBS and identifying those patients who are at risk of severe kidney disease. A number of challenges must be overcome before therapeutic intervention for BBS renal disease can become a realistic prospect.

A primary challenge highlighted by this study is the prenatal onset of structural aberrations in many patients and early onset of renal dysfunction in patients who develop severe kidney disease. Prospects for therapeutic intervention are most realistic if intervention can be implemented before the onset of severe kidney disease. In some cases this will require very early diagnosis and treatment-potentially prenatal- which would severely limit the number of patients eligible for therapy.

A number of targeted, non-genetic therapies are under investigation for ADPKD that could potentially be applicable to renal disease in BBS. However, pre-clinical trials are limited by the lack of BBS mouse models adequately reflecting the renal disease observed in humans. Firstly, not all mouse models develop kidney disease. Those that consistently do are generally knockout models, which do not reflect the genetic aberrations seen in humans, and are therefore less likely to be appropriate candidates for assessing the effect of genetic therapies. The *BBS1* p.Met390Arg knock-in mouse model mimics many of the human phenotypic features including retinal dystrophy, male infertility and obesity. However, renal disease is not consistently observed[147] which is in keeping with the observation from this study that significant renal disease is rarely a feature in patients carrying *BBS1* p.Met390Arg mutations. None of the available mouse models express the variety of kidney abnormalities observed in BBS patients in this study, which may limit their use as good biological models for therapeutic intervention. Clear practical and ethical issues limit the availability of human renal tissue to be studied to identify abnormalities of ciliary structure or function which could potentially identify

cellular aberrations that could be targeted for therapies or used as therapeutic markers. This challenge may be overcome as expertise in transforming induced pluripotent stem cells into various renal cell types and kidney organoids progresses[148-151].

The lack of a reliable BBS disease biomarker for evaluating biological effectiveness would be a potential challenge in testing novel therapies. A recent proteomics study highlights initial work to identify a urinary marker of renal disease in BBS. Promising results demonstrated 42 over- or underexpressed proteins on analysis of patients with BBS compared to controls[152]. These proteins were mostly involved in fibrosis, cell adhesion and extracellular matrix organisation, and in addition the presence of urine fibronectin correlated with decline in eGFR[152]. This could potentially hail the development of a non-invasive diagnostic marker for renal dysfunction and therapeutic effectiveness in the future.

A further challenge is that BBS renal disease is both primary and secondary in nature, and non-genetic risk factors, primarily obesity, hypertension and diabetes mellitus are likely to have a significant effect on adult onset renal disease in BBS patients. The impact of aggressive management of these non-renal factors on the prevalence of CKD and natural history of renal disease in BBS remains to be explored in the future.

In conclusion, this study has made a significant contribution to our understanding of the structural and functional renal deficits in BBS, identifying specific risk factors for CKD, as well as proposing management pathways for renal disease based on risk stratification. A number of challenges must be overcome before therapeutic intervention for BBS renal disease can be implemented, but ongoing work to develop targeted therapies for ADPKD may be relevant to BBS renal disease due to the likely overlap in molecular pathogenesis.

4 Bardet-Biedl syndrome and the eye: Understanding the phenotype, predicting outcome, and planning for future therapies

"When will my child go blind and when will there be a cure for blindness in BBS?"

4.1 Introduction

Visual deterioration is the most common diagnostic handle prompting investigation for BBS and is present in more than 90% of patients according to published evidence[3]. It is the second most common cause of syndromic retinal degeneration after Usher syndrome[153]. Visual loss characteristically presents as an atypical pigmentary retinal dystrophy with early macular involvement[154,155]. Deterioration in dark adaptation (nyctalopia) typically occurs in the first decade of life[156] and is the first sign of decline. Most patients are registered blind by the second or third decade of life[157]. Following the onset of night blindness, patients with BBS usually develop decreased peripheral vision due to a loss of rod photoreceptors followed by loss of cone photoreceptors leading to diminished colour vision[157]. The macula is often affected early on in the disease pathogenesis[153]. The onset of visual deterioration is highly variable.

4.1.1 Retinal degeneration is the most consistent feature seen in Bardet-Biedl syndrome and several different types are seen in the patient population

Although the reported frequency of retinal dystrophy in BBS is 90%, this is based on cohorts of patients with a confirmed clinical diagnosis but not necessarily accompanied by molecular confirmation. In practice, the UK national BBS clinics have not seen any adults with both molecular and clinical confirmation of the diagnosis who did not at least display the early stages of retinal dystrophy on ophthalmoscopy.

Several different types of retinal dystrophy in BBS have been reported. Most patients present with a rod-cone dystrophy[158], whereby there is a primary loss of the light sensitive rods followed by loss of cones, the colour perception photoreceptors. A smaller proportion of patients present with cone-rod dystrophy where cones are lost followed by rods[159]. Initial loss of vision can be central or peripheral. Some patients present very early on with degeneration in both rods and cones and this can best be classified as severe global retinal deterioration[158]. Other patients have been reported to have choroidal dystrophy[158,159].

4.1.2 Photoreceptors are highly specialised primary cilia

Photoreceptors arise from a pool of dividing multipotent progenitor cells[160] and develop as a result of a budding protoplasm extending beyond the outer limiting membrane forming the inner segment of the photoreceptor[159]. The bud contains the endoplasmic reticulum, golgi apparatus, mitochondria and centriole. The centriole migrates to the apical side of the inner segment and the cilium begins to grow from the basal body. Subsequently, the differentiation of the photoreceptor beyond that of a standard primary cilium commences. The distal portion of the cilium expands and forms disorganised vesicular sacs in the outer segment of the photoreceptor, which are later organised into stacked discs. The discs optimise the total area available for the photopigment protein opsin thereby allowing high sensitivity to light[160]. Discs are formed by the evagination of the plasma membrane[159]. In rod photoreceptors the discs are separate from the plasma membrane, whereas in cone photoreceptors they are continuous with plasma membrane[159].

Additional features that set this highly specialised primary cilium apart from other primary cilia is that distally from the basal body extending into the outer segment the

standard 9+0 configuration made up of microtubule doublets converts into a 9+0 singlet configuration[160]. Figure 4.1 illustrates the structure of the human photoreceptor.

It is noteworthy that although several genes associated with ciliopathies are involved in ciliogenesis, no ciliopathy is associated with a lack of photoreceptor development, suggesting that perhaps compensatory mechanisms are available for ciliogenesis but not for maintenance and function of the cilium.

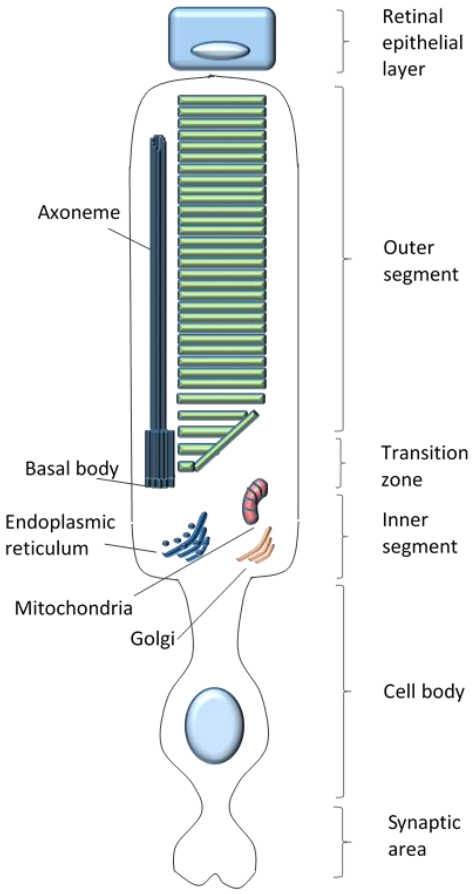


Figure 4.1 Schematic of a human rod photoreceptor
The photosensitive outer segment has stacks of discs and links with the inner segment through the cilium. The doublet microtubules of the cilium become singlet microtubules in the outer segment.

4.1.3 Visual decline is a product of mislocalised rhodopsin

Retinal decline is thought to relate to a defect in photoreceptor intraciliary transport[6] resulting in rhodopsin mislocalisation. The biosynthetically active inner segment of a photoreceptor contains the major trafficking and metabolic machinery[161]. The light sensitive outer segment contains the phototransduction apparatus but cannot synthesise the cell components required for cell transduction[154]. Proteins and membranes flow from the inner to the outer segments through IFT mechanisms[154].

Rhodopsin usually localises to the outer segment of the photoreceptor and plays a critical role in the first step of phototransduction[157]. However, in BBS mouse models, rhodopsin primarily localises to the inner segment and the outer nuclear layer[162] and on histological examination, the outer segment appears disorganised[163]. As a result, apoptosis ensues with increasing loss of the outer segment, inner segment and outer nuclear layer[164]. Hsu *et al* showed that in a BBS mouse model where temporal restoration of the BBSome is possible, malformed outer segment discs can be replaced by functioning outer segments in immature photoreceptors[162].

Quantitative proteomics research examining the outer segment proteome in a *Bbs17* mouse model demonstrated accumulation of 138 non-outer segment proteins[163]. This included the SNARE complex of which one component, STX3, has been associated with rhodopsin trafficking in photoreceptors[163]. It has been hypothesised that BBS proteins are required for protein trafficking from the outer segment to the cell body, and that toxic accumulation of proteins may be a crucial component in BBS photoreceptor death[157].

4.1.4 Rationale

Visual deterioration is one of the most devastating aspects of BBS. Most patients are eventually registered blind but the variation in age at diagnosis and registration of blindness is considerable. Identifying genotype-phenotype correlations, the degree of variability of onset and timeframe of decline in visual function will give the necessary insight into the natural history and variability in phenotype required in anticipation of clinical trials for gene therapy.

4.2 Results

4.2.1 Overview of the population

Two hundred and thirty five out of 400 patients responded to the survey. The mean age of responders was 30.5 years (range: 0-59) and the male to female ratio was 49%: 51%.

The survey was distributed to all patients attending the UK national Bardet-Biedl syndrome clinics in London and Birmingham. Molecular confirmation of the diagnosis had been achieved in 163 patient responders (70%) primarily on sequencing the four most common mutations in BBS: *BBS1* p.Met390Arg, *BBS2* p.Tyr24*, *BBS2* p.Arg275*, *BBS10* p.Cys91Leufs*5. Molecular confirmation of the diagnosis was achieved in additional patients with mutations in *BBS4*, *BBS7*, *BBS8*, *BBS9*, *BBS12* using a 13 gene panel.

All patients were asked to record the absence/ presence of nyctalopia (night blindness), diagnosis of rod-cone dystrophy (RCD) and registration of blindness as well as the age at which these features were first noted/ diagnosed. One hundred and forty one patients reported night blindness, 150 patients reported a diagnosis of rod-cone dystrophy and 117 patients reported being registered blind.

One hundred and thirty patients identified the age at which their night blindness had started. One hundred and thirty seven recorded the age at which they had been diagnosed with rod-cone dystrophy and 117 patients recorded the age at which they were registered blind.

Figure 4.2 illustrates the distribution of genotypes amongst patient responders. Of note, sixty out of 88 patients with *BBS1* mutations were homozygous for *BBS1* p.Met390Arg and 20 were heterozygous for *BBS1* p.Met390Arg.

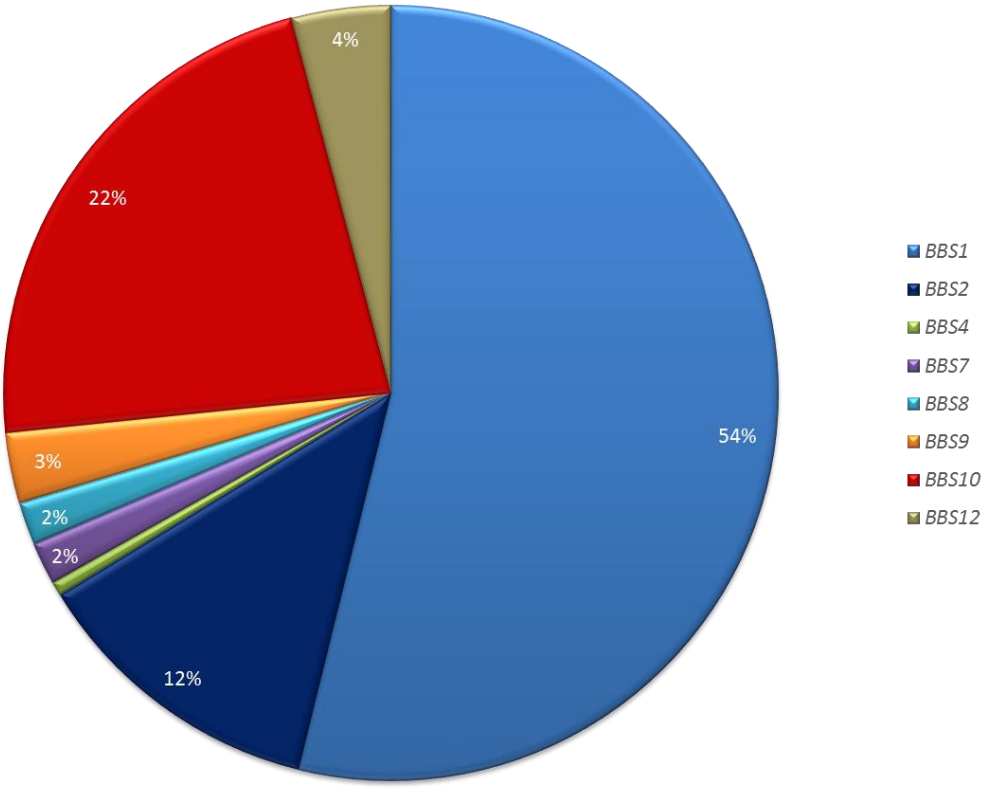


Figure 4.2 Distribution of genotypes of questionnaire responders
The majority of patient responders had mutations in *BBS1* followed by mutations in *BBS10*.

4.2.2 Patients with mutations in *BBS1* are older when they develop visual deterioration than patients with mutations in other genes.

Age at visual deterioration was assessed for all three indicators of decline: night blindness, rod-cone dystrophy and registration of blindness. Sample sizes allowed for statistical evaluation of the three most common genotypes *BBS1*, *BBS2* and *BBS10* and

for three categories of mutation combinations (missense/ missense, missense/ truncating and truncating/ truncating).

Patients with mutations in *BBS1* developed visual deterioration later than patients with mutations in *BBS2* and *BBS10* across all three deterioration indicators although this was only statistically significant for age at diagnosis of rod-cone dystrophy ($p=0.004$ and $p<0.0001$ respectively) (figure 4.3). Patients with mutations in *BBS2* appeared to maintain their visual function for longer than patients with mutations in *BBS10* although this was not statistically significant.

Patients with missense mutations maintained their vision for significantly longer than patients with missense/ truncating and truncating/ truncating mutations across all three deterioration indicators (all p values <0.01, see figure 4.3).

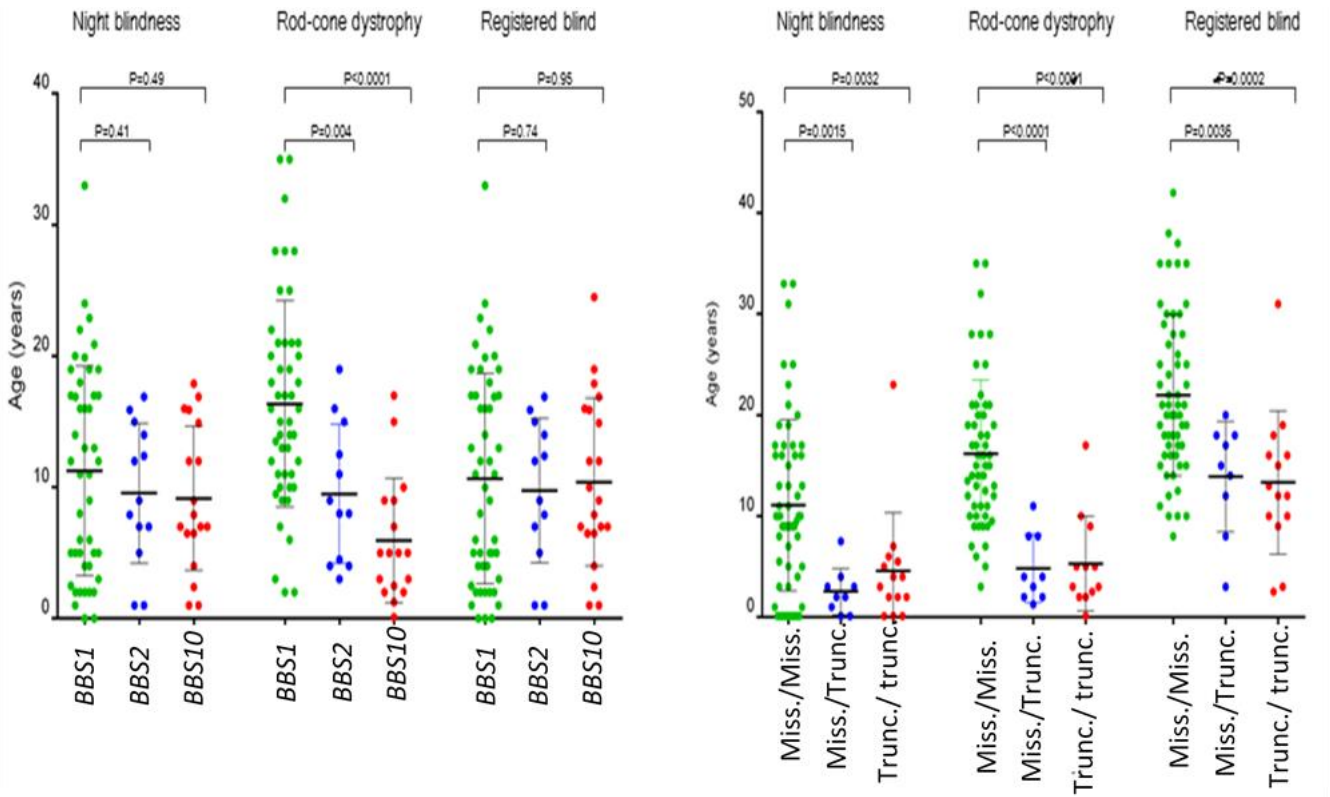


Figure 4.3 Visual decline correlates with genotype

Visual decline correlates with genotype Patients with *BBS1* mutations and missense mutations have visual decline later than patients with mutations in *BBS2* and *BBS10* and other mutation combinations. RCD: Rod-cone dystrophy.

The medical records were examined for patients with both severe and mild ocular phenotypes including patients who were registered blind before the age of 10 and patients who were not registered blind before the age of 20.

Genotype and mutation type were assessed for patients who were registered blind before the age of ten. Table 4.1 highlights the genotypes, mutation type and age at visual deterioration in these patients and demonstrates that these patients have an overwhelming prevalence of truncating mutations. No patients with mutations in *BBS1* were present in this cohort.

An assessment of patients who were not registered blind beyond the age of 20 revealed that the majority of these patients had the common homozygous p.Met390Arg missense mutation in *BBS1*. The remaining patients had mutations in *BBS10* and evidence of more severe disease on fundoscopy (table 4.2).

Table 4.1 Genotype and mutation type of patients registered blind before the age of 10

Gender	***Genotype***	***Mutation type***	***Age at night blindness***	***Age at diagnosis of RCD***	***Age at registration of blindness (*)***
M	*BBS2*	p.Tyr24*/ p.Arg275*	2	3	3
F	*BBS2*	p.Arg275*/p.Arg275*	?	3	3
F	*BBS2*	p.Arg275*/p.Arg275*	?	?	8
F	*BBS9*	p.Ile41Ser/p.Tyr186Cys	5	5	6
M	*BBS10*	p.Thr79NAsnfs*7/ p.Thr79Asnfs*7	?	2.5	2.5
F	*BBS10*	p.Ser653Ilefs*4/ p.Ser653Ilefs*4	2	2	3
F	*BBS10*	p.Tyr186Cys/ p.Tyr186Cys	?	?	8
M	*BBS10*	p.Cys91Leufs*5/ p.Cys91Leufs*5	2	3	9
M	*BBS12*	p.Phe372*/ p.Arg674Cys	5	5	8
F	*BBS12*	p.Gln58*/ p.Gln365Argfs*18	2	6	9

*All patients had end stage findings of retinal dystrophy on fundoscopy

Table 4.2 Patients aged 20 or over who are not registered blind

Age	*Gender*	*Genotype*	*Mutation type*	*Age NB started*	*Age RP diagnosed*	*Fundoscopy findings*
31	F	*BBS1*	p.Met390Arg/ p.Met390Arg	13	13	Intraretinal pigment migration in the midperiphery. Atrophy of retina but island of normal retinal at the fovea. Pale optic discs.
20	F	*BBS1*	p.Met390Arg/ p.Met390Arg	N/A	N/A	Foveal atrophy and minimal intraretinal pigment migration.
22	M	*BBS1*	p.Met390Arg/ p.Met390Arg	N/A	N/A	Foveal atrophy and minimal intraretinal pigment migration.
28	F	*BBS1*	p.Met390Arg/ p.Met390Arg	N/A	N/A	Minimal macular atrophy.
53	F	*BBS1*	p.Met390Arg/ p.Met390Arg	46	52	Atrophy of the macula. Mild pigmentary disturbance of the retinal periphery.
38	F	*BBS1*	p.Met390Arg/ p.Met390Arg	27	32	Early atrophic signs and some pallor at the optic discs. Peripheral changes showed a mild to moderate degree of pigmentary disturbance.
20	F	*BBS1*	p.Met390Arg/ p.Met390Arg	N/A	19	Subtle peripheral changes discrete abnormalities at the foveal centre.
43	F	*BBS1*	p.Met390Arg/ p.Met390Arg	45	45	Normal fundal examination. Mild colour vision defect.

28	M	*BBS1*	p.Met390Arg/ p.Met390Arg	23	23	Sparse pigmentary changes and early macular atrophic changes.
21	F	*BBS10*	p.Cys91Leufs*5/ p.Ala216Glyfs*	5	1	Heavy pigmentation. Pale optic discs
23	F	*BBS10*	p.Arg95Ser/ p.Val707*	8.5	13	Early signs of pigmentation. Pale discs. Changes on the macula bilaterally.
27	M	*BBS10*	p.Thr177CYs/ p.Thr177Cys	N/A	5.5	Small central area of limited vision. Atrophy throughout the retinal.

4.2.3 Patients with mutations in *BBS1* are statistically more likely to experience a slower decline in visual function than patients with mutations in *BBS2* and *BBS10*.

The rate of visual function decline was assessed by measuring the number of years between the onset of night blindness to the age at registration of blindness. The mean age of decline for patients with mutations in *BBS1* was 12.4 years (SD: 8.6), for patients with mutations in *BBS2* it was 9.8 years (SD: 5.2) and for patients with mutations in *BBS10* it was 10.4 years (SD: 6.2). The age range for patients with mutations in *BBS1* was larger than for patients with mutations in the other genes. There was a statistically significant difference in deterioration on comparing *BBS1* versus *BBS2* (Mann-Whitney U test: p=0.002) and *BBS1* versus *BBS10* (Mann Whitney U test: p=0.0001) but not *BBS2* versus *BBS10*.

On comparison of mutation types the median age of deterioration for patients with two missense mutations was 10.9 (SD: 7.9), for missense/ truncating mutations 11.3 (SD: 5.4), and two truncating mutations 8.76 (SD: 3.9). There was no statistically significant difference in rate of deterioration of visual function between the three mutation groups.

4.3 Discussion

This is the largest study assessing genotype-phenotype correlations of visual function in BBS. The frequency of genotypes in this cohort is similar to that observed in the renal study suggesting that the responders to this questionnaire are representative of the group as a whole.

Patients with mutations in *BBS1* maintain their vision in all three visual function modalities for longer than patients with mutations in *BBS2* and *BBS10,* although this is only statistically significant on comparison of the age at diagnosis of rod-cone dystrophy.

On assessment of mutation type patients with two missense mutation maintained their vision for significantly longer than patients with missense/ truncating or two truncating mutations across all deterioration parameters. Mockel *et al* attempted to identify a genotype-phenotype correlation between patients with *BBS1* (n=5) and *BBS10* (n=3), but no clear correlation was evidenced between genotype and retinal degeneration as seen on fundus photography[159]. A study of 37 patients by Daniels *et al*[165] demonstrated that patients with mutations in *BBS1* had better visual function and higher electroretinogram amplitudes than patients with mutations in other genes. This study also assessed mutation type in multivariable regression analysis including age and genotype, but this did not reveal a mutation type effect. However, since the majority of patients with *BBS1* mutations in this study had at least one missense mutation this may represent a type II statistical error since mutation type appeared to exert a significant effect on phenotypic outcome.

It was not possible to conduct multivariable analysis on this patient cohort due to the skewed representation of mutation types within each genotype –primarily the common p.Met390Arg missense mutation in *BBS1*, nonsense mutations in *BBS2* and frameshift mutations in *BBS10*.

The rate of deterioration revealed a statistically significant difference between genotypes but not mutation type. It was notable that patients with mutations in *BBS1* and missense mutations demonstrated large differences in deterioration rates (range: 0-33 years for both groups). This may reflect the large number of missense mutations found in *BBS1*.

There is a clear genotype-phenotype and in particular a mutation type-phenotype correlation in the visual function of patients with BBS. Patients with missense mutations

and mutations in *BBS1* retain visual function for longer than those with nonsense or frameshift mutations and mutations in *BBS2* and *BBS10*. This may occur as a result of mutation type, genotype or more likely a combination of both factors where patients with *BBS1* p.Met390Arg, on average, have a hypomorphic phenotype.

It is noteworthy that all patients with molecular confirmation of their diagnosis had symptoms or signs of rod-cone dystrophy. This study indicates that rod-cone dystrophy may be a fully penetrant feature of BBS. The remaining 10% of patients reported in the literature may fulfil the clinical criteria for BBS by exhibiting other primary and secondary features. However, the other features are less specific to BBS and may be occur as a result of other syndromes.

After the end of this study a number of patients have been seen in the national BBS clinics referred with *BBS1* mutations but displaying only rod-cone dystrophy and none of the other features of BBS (own unreported observations).

Limitations The limitations of this study pertain primarily to the self–reporting of night blindness, diagnosis of rod-cone dystrophy and registration of blindness which introduces significant bias. The data obtained might have been verified through access to GP medical records, although given the number of patients spread across the UK this would be logistically impracticable.

In some patients nyctalopia starts before they are able to verbalise their symptoms and as a result there may be a delay before this is noted by parents.

A limitation that compounds the self-reporting is that age at diagnosis of rod-cone dystrophy and the age at registration of blindness may be delayed depending on patient circumstances and individual preference and therefore some of the patients' results may be inadvertently skewed.

It was not possible to assess the hypomorphic effect of the *BBS1* p.Met390Arg since the majority of patients with mutations in *BBS1* are at least heterozygous for this common allele.

Since the results of this survey were analysed in 2013, the diagnostic rate has improved from 70% to 80% and hence 10% of patients who were undiagnosed at the point of analysis have subsequently acquired molecular confirmation.

Future directions As our knowledge of the BBS phenotype continues to expand it is becoming increasingly clear that the current clinical criteria for diagnosis[49] capture only a selected sub-group of patients. Those patients without evidence of rod-cone dystrophy are unlikely to gain molecular confirmation of their diagnosis and conversely patients with non-syndromic rod-cone dystrophy may have mutations in BBS. These findings may call for a review of the clinical criteria. This could include an evidence-based age cut-off for the development of rod-cone dystrophy.

One of the main challenges for the future is devising a practical approach to assessing visual deterioration and predictive tools to understand the decline in visual function in BBS. Visual loss in BBS is inherently complex. Not only can different patients have different forms of retinopathy but even within the same retinopathy symptoms can manifest differently. Some patients may retain only a small proportion of healthy photoreceptors but if these are centrally located and macular vision is retained then significant overall visual function is preserved. Conversely, patients may have retained a significant photoreceptor function but if macular photoreceptors have apoptosed the overall functional vision will be poor. Several factors need to be considered to assess the overall visual function including visual acuity, presence of visiospatial disturbance and visual field.

Understanding the ophthalmological phenotype and natural history in BBS is a crucial prerequisite for future genetics therapeutics trials. Understanding the natural history is required to assess the efficacy of any novel therapy. Recent unpublished work with the patient group at Great Ormond Street Hospital demonstrates that children who are homozygous for *BBS1* p.Met390Arg have a distinctive retinal pattern correlating with later onset visual deterioration on fundus autofluorescence, correlating with the findings in this study. Several potential therapeutic options exist; for ciliopathies exon skipping therapy for LCA is in development[166] as well as readthrough therapy for Usher syndrome[86] and Retinitis Pigmentosa[87].

Ongoing research to develop gene therapy for *BBS1*[61,80] and, prospectively other genotypes, looks promising and will hopefully result in clinical trials in the coming years.

5 Understanding obesity in Bardet-Biedl syndrome

"Why can I not stop my child from gaining weight- will there ever be a treatment for it?"

5.1 Introduction

Obesity is a metabolic disorder characterised by an imbalance between energy intake and expenditure[56]. Assessing obesity in cohorts is usually done through Body Mass Index (BMI) scores for adults and BMI-Standard Deviation Scores (BMI-SDS) for children. The advantage of using BMI scores is the relative ease with which these can be calculated on a large scale. It is not an ideal measure of adiposity[167] but correlates well with other markers of obesity such as waist circumference and waist-hip ratio[168]. BMI-SDS scores in children are advantageous as the SDS scale is linear and sex-independent making results comparable across age groups and between genders[169].

5.1.1 Obesity and genetics

There are three principal forms of obesity: common obesity, monogenic obesity and syndromic obesity.

By far the most frequent type is common or complex obesity occurring as a factor of environmental and genetic predispositions. Twin and adoption studies demonstrate that the heritability of BMI is around 40-70%[170,171]. Genome Wide Association Studies (GWAS) have identified up to 97 susceptibility loci for complex obesity accounting for 2.7% of variability in BMI[172].

Around 3-6% of the obese population have monogenic non-syndromic obesity. It is clear that even in cases of monogenic obesity environmental factors still play a significant role[173]. Mutations in genes in the leptin pathway constitute the most common cause of monogenic obesity[173].

The third, much less common type of obesity, is syndromic obesity. This is characterised by an association with a distinct clinical phenotype such as learning difficulties, dysmorphic features, and other organ specific abnormalities[173].

Around 25 genetically elucidated forms of syndromic obesity have been identified ranging from chromosomal deletions to the commonest form of syndromic obesity: Prader-Willi Syndrome (PWS), which has an incidence of around 1:25,000[173].

Not all genetic obesity syndromes described in the literature have been named or fully molecularly elucidated[174]. One meta-analysis identified up to 79 potential obesity syndromes where only 54% had been characterised but not named[174].

5.1.2 Appetite regulation, obesity and genetics

Appetite regulation occurs both peripherally through the secretion of gut hormones and centrally through the hypothalamic melanocortin-4 (MC4) pathway. Peripherally, leptin, pancreatic polypeptide (PP) and insulin are long-acting regulators released into the blood in proportion with the amount of body fat[175]. If body fat stores decrease, so does secretion of leptin and insulin, in turn signalling to increase appetite[175]. Short term regulators include cholecystokinin, gastric peptide ghrelin (appetite accelerators) and peptide YY (an appetite suppressor). Levels of these hormones rise and fall in tandem with gastric and small bowel distension. The levels of short term regulators are also influenced by long term regulators leptin and insulin.

Peripheral gut hormones act centrally on the appetite centre in the brain situated in the arcuate nucleus (ARC)[176]. This region contains a group of first order neurons receiving peripheral metabolic signals. These include the orexigenic peptides NeuroPeptide Y (NPY) and Agouti –Related Peptide (AgRP) and the anorexigenic Pro-OpioMelanoCortin

(POMC)[177] and Cocaine and Amphetamine Regulated Transcript (CART)[178]. The orexigenic post-transcriptional product of POMC- alpha melanocyte-stimulating hormone (α-MSH)- acts on Melanocortin 3 and 4 receptors (MC3R, MC4R), which are second order neurons competing with anorexigenic AgRP for synaptic space. The resultant regulation of catabolic hormones including corticotrophin-releasing hormone, thyrotropin- releasing hormone, somatostatin, vasopressin and oxytocin regulate energy homeostasis. In addition, third order neurons also control sympathetic outflow to the liver and adipose tissue promoting increased fatty acid oxidation and lipolysis[178]. Other regulators such as Ciliary Neurotrophic Factor (CNF) and Brain Derived Neurotrophic Factor (BDNF) also play a role in appetite homeostasis. Figure 5.1 summarises the current understanding of appetite regulation.

Monogenic obesity occurs principally through mutations in genes involved in the leptin-melanocortin pathway[173,179]. Mutations in the Melanocortin-4-Receptor (MC4R) account for the vast majority[180]. Monogenic obesity is even more prevalent in countries with high levels of consanguinity[179,180] accounting for up to 30% of the obese population[179,181].

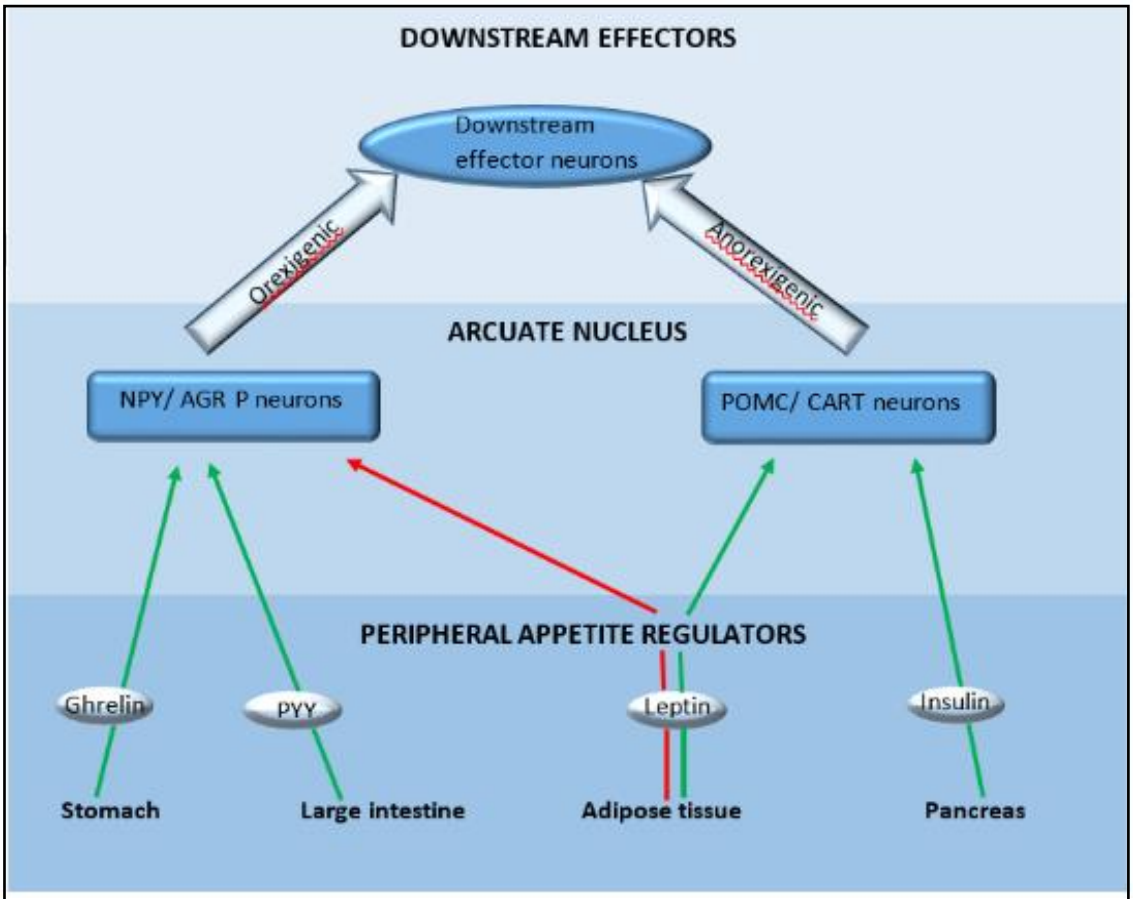


Figure 5.1 Appetite regulation takes place in the hypothalamus

Leptin and insulin have an anorexigenic effect and result in the reduction of food intake, whereas Ghrelin and PYY have orexigenic and anorexigenic effects respectively. Green arrows denote positive regulation and green arrows denote negative regulation.

5.1.3 Managing genetic and syndromic obesity

Several treatment options for people with obesity exist. Mean weight loss associated with each type of intervention varies considerably. Lifestyle interventions result in a mean weight loss of 3-8%, pharmacotherapy and lifestyle intervention around 10-13%, gastric banding 16% and gastric bypass 32%[182,183]. Each approach has benefits and drawbacks, but it is clear that lifestyle alone is unlikely to have a sufficient effect on the severe obesity that prevails in syndromic obesity including BBS.

5.1.4 Pathogenesis of obesity in the ciliopathies and Bardet-Biedl syndrome

Although individually rare the ciliopathies account for a large proportion of the 25 known forms of syndromic obesity and include BBS (prevalence 1:100,000), Alstrom Syndrome (prevalence 1:1,000,000), Borjeson-Forssman-Lehman syndrome (prevalence <1:1,000,0000), Carpenter syndrome (prevalence <1:1,000,0000), Cohen Syndrome (prevalence <1:1,000,0000) and Mental Retardation- Truncal Obesity-Retinal Dystrophy-Micropenis (MORM) syndrome (prevalence <1:1,000,0000) [177].

The high prevalence of obesity (70-90%) in BBS as well as other ciliopathies suggests that cilia play a significant role in appetite regulation[184]. Although birthweight is often normal in people with BBS, children usually develop marked obesity, which becomes truncal in nature in adolescence and adulthood[3]

There is limited research available on obesity in humans with BBS but it has been suggested that hyperphagia, rather than specific food seeking behaviour (such as is characteristically seen in PWS), is the major driver in developing obesity[184]. Anecdotal evidence from the UK national BBS clinics does, however, suggest that many families experience temper tantrums when food is withheld in a similar fashion to that observed

in families affected with PWS. One study suggested that lower activity levels are prevalent in patients with BBS compared to matched controls[185].

Several lines of investigation support a role for cilia in normal energy metabolism. Artificial induction of short cilia by siRNA mediated knockdown of *Ift88* and *Kif3a* in adult mice led to decreased energy expenditure and weight gain[176]. The anorexic response to leptin and insulin was also reduced in these mice[176]. Interestingly, short cilia are also observed in the hypothalamus of diet-induced obese mice[177].

Evidence from BBS knockout mice suggests that BBS is associated with hyperleptinaemia and concomitant leptin resistance[103]. The leptin resistance may be mediated by aberrant cilia function that affects the leptin receptor or its downstream targets[103]. The molecular mechanism of leptin resistance in BBS is poorly understood. However, investigations in *Bbs* mice demonstrate that the BBS1 protein interacts with the leptin receptor and is involved in trafficking the receptor to the cell surface membrane[103]. Tissue-specific deletion of *BBS17* in a mouse model has demonstrated that deletion of *BBS17* in the hypothalamus, but not adipose tissue eliminates phosphorylation of Stat3 in the Leptin signalling pathway thus inducing the obesity phenotype[186]. Furthermore, ciliary localisation of the hypothalamic appetite stimulating hormone receptor Melanin-Concentrating Hormone Receptor 1 (MCHR1) is disturbed in Bbs2 and Bbs4 deficient mice[187].

A study of 50 individuals with BBS demonstrates that mean leptin levels in BBS patients are around two fold higher than in matched controls[188]. On genotype-phenotype analysis patients with mutations in *BBS1* had significantly lower BMI-SDS scores than *BBS10* patients and non-significant trends towards lower leptin levels[188]. The study also

investigated adiposity and found that there was no significant difference between BBS patients with BMI matched controls[188].

An alternative theory suggests that altered adipose tissue development may also contribute to obesity in BBS. Primary cilia are transiently present during differentiation of cultured pre-adipocytes. Marion et al[189] showed that siRNA mediated inhibition of *Bbs10* and *Bbs12* expression in differentiated pre-adipocytes impaired ciliogenesis as well as activating adipogenesis signals through nuclear accumulation of peroxisome proliferator-activated receptor γ (PPAR-γ) and the GSK-3β pathway.

5.1.5 Managing obesity in Bardet-Biedl syndrome

No specific intervention exists for obesity in BBS or the other ciliopathies. A number of clinical trials have been conducted in PWS, the most common form of syndromic obesity. One open-label six month clinical trial examined the effect of Exanatide, a Glucagon-Like-Peptide-1 (GLP-1) agonist associated with modest weight loss in the general obese population, and demonstrated a good effect on appetite and HbA1c in PWS but no effect on weight, BMI-SDS score or adiposity[190]. Another study assessed the effect of Methionine AminoPeptidase 2 (MetAP2) inhibitor, Beloranib in a 26 week randomised double blind placebo controlled phase 3 trial. Although a significant change in appetite and body weight was observed, further developments in Beloranib have been discontinued due to serious adverse effects[191]. A randomised trial examining the effect of ghrelin analogue AZP-531 showed significant effects on appetite but not body weight [192].

Iepsen et al[180] trialled GLP-1 agonist Liraglutide in 14 patients with MC4R mutations and 28 matched controls for three months and found that participants in both groups lost

6% of their body weight. This could be a result of one of two effects: either it is because Liraglutide is exerting a poorly understood effect on the ARC neurons or it is possible that optimising the GLP-1 pathway can compensate for another poorly functioning appetite regulation pathway[193].

A newly developed MC4 Receptor Agonist, Setmelanotide, has been tested in a phase 2 trial in five patients with BBS[194]. Initial results are promising and indicate an impressive potential response accompanied by a drop in appetite[194].

5.1.6 Rationale

Previously only small cohort studiesrefle have been conducted to decipher the obesity phenotype in BBS. There is no evidence-based consensus strategy for managing obesity in BBS specifically or obesity syndromes in general. This study assesses the obesity phenotype and natural history in a large BBS cohort of children and adults as well as the efficacy of the current 18 monthly dietetics intervention.

5.2 Results

Patient data were assessed separately for children and adults reflecting differential anthropomorphic measurements and dietetics input.

Patients participating in this study attended the UK national BBS clinics in London during a six year period (2010-2016). The age range of patients attending the BBS clinics varied from newborn to 60 years old. Only patients over the age of two were included in the study since BMI-SDS is unreliable in children who are under the age of two.

Patients were included in the study if they had both clinical and molecular confirmation of BBS.

5.2.1 Children

Seventy-two children with molecularly confirmed BBS were included in the study. Twenty- three patients had mutations in *BBS1*, 15 in *BBS10* and the remaining patients had mutations in other known BBS genes. The distribution of genotypes is shown in figure 5.2. The mean age(SD) of attenders was 8.6(4.3) years. The mean number of years between clinic attendances was 1.65 years.

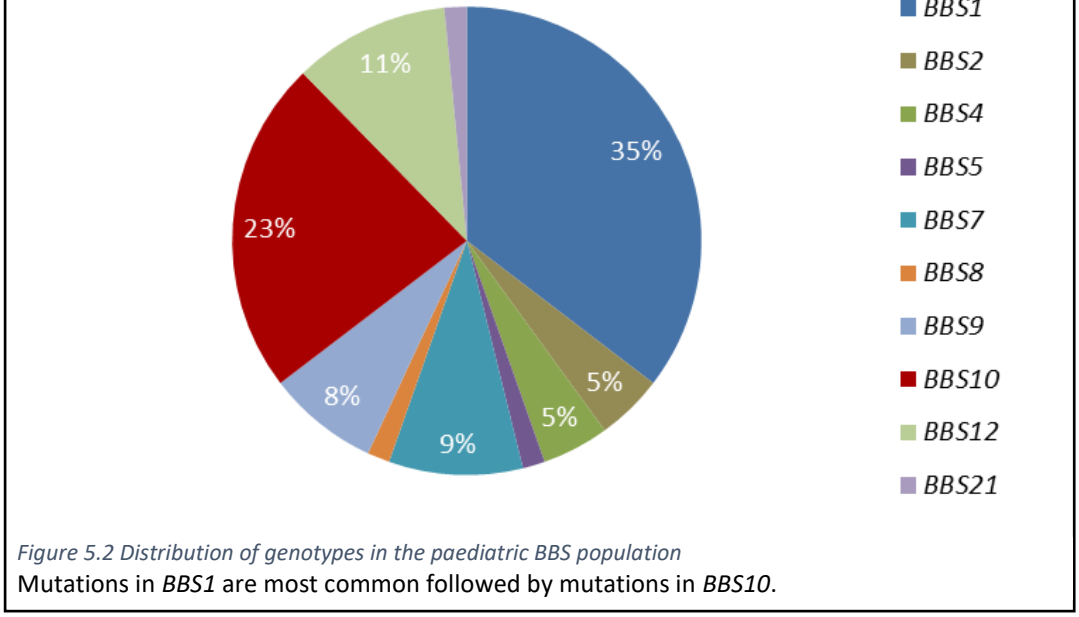


Figure 5.2 Distribution of genotypes in the paediatric BBS population
Mutations in *BBS1* are most common followed by mutations in *BBS10*.

5.2.1.1 The majority of children are obese at first clinic attendance

Children were classified according to obesity category; a BMI-SDS score below 1.33 was classified as normal, 1.33-1.99 overweight, 2-2.66 obesity class I, 2.67-3.49 obesity class II and above 3.5 obesity class III[195].

The majority of children were obese at the first visit with only 5% having a weight within normal range. Eighty-three per cent of children were obese at first clinic attendance. Figure 5.3 demonstrates the BMI-SDS distribution of children attending the paediatric

clinics for the first time. Figure 5.4 compares obesity categories in patients with mutations in *BBS1* versus *BBS10* demonstrating that severe obesity is more prevalent in patients with *BBS10* mutations.

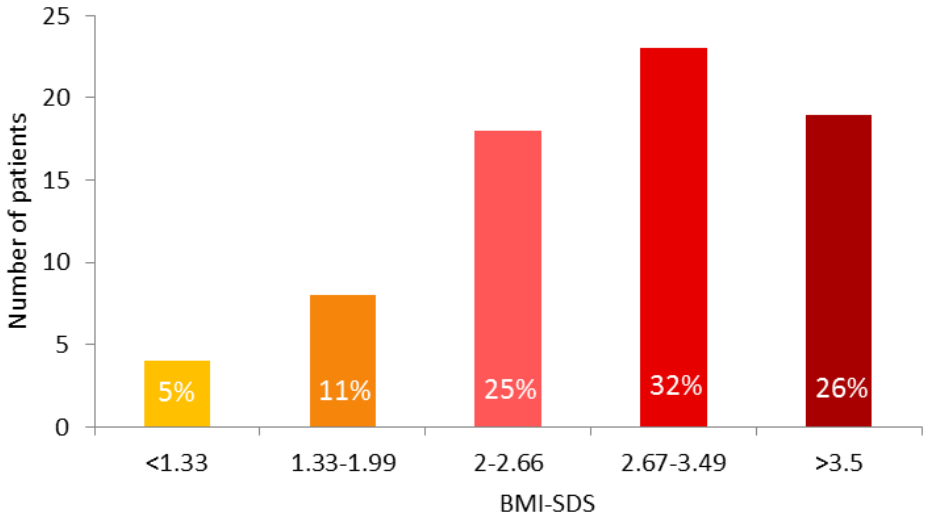


Figure 5.4 The majority of children with BBS are obese

Percentages denoted in each column. N=72. Only 5% of patients have a normal BMI. Eighty-three per cent of patients are classed as obese.

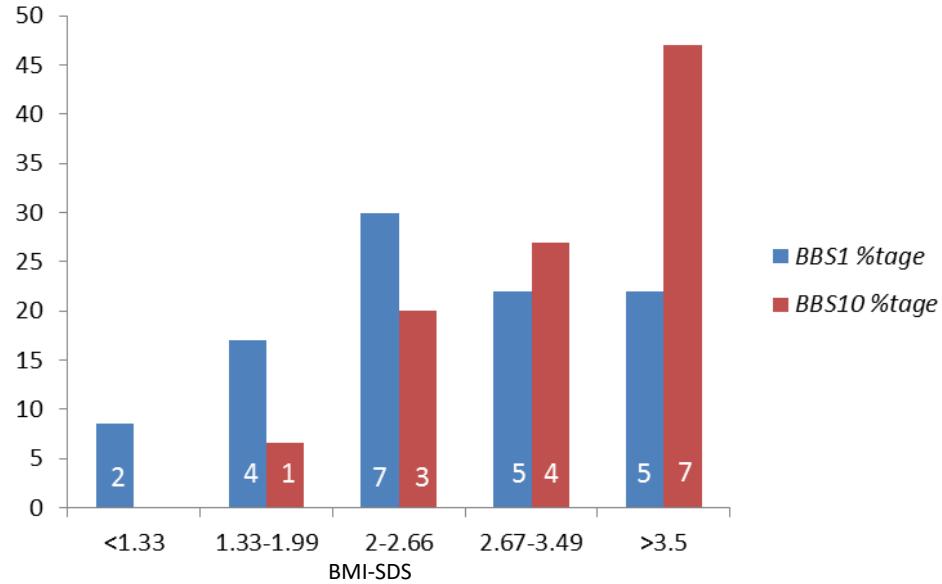


Figure 5.3 Children with mutations in BBS10 are more likely to be severely obese

This figure demonstrates the percentage of patients in different BMI categories within each genotype. Absolute numbers are given in each column.

5.2.1.2 Weight varies significantly among paediatric patients and correlates with genotype

Weight is highly variable at the first visit especially before the age of six in patients with mutations in genes other than *BBS1* as demonstrated in figure 5.5. The majority of patients have a weight-SDS between 1 and 4 (figure 5.5a). Height-SDS is primarily within the normal range, although a significant number of patients are taller than +2 SDS. The taller patients primarily have mutations in *BBS1*. Very few patients with mutations in *BBS1* are below the normal range for height (figure 5.5b). The correlation coefficient between Height-SDS and Weight-SDS was R=0.58. Figure 5.5c demonstrates that there is considerable variation in BMI-SDS –particularly in children under six years. Patients with *BBS1* mutations usually have a BMI-SDS between 1 and 4 and that for older children this is typically between 2 and 3. Patients with *BBS10* mutations typically have higher BMI-SDS scores than *BBS1* patients (unpaired T-test: p=0.02). There was no statistically significant difference in BMI-SDS between mutation types.

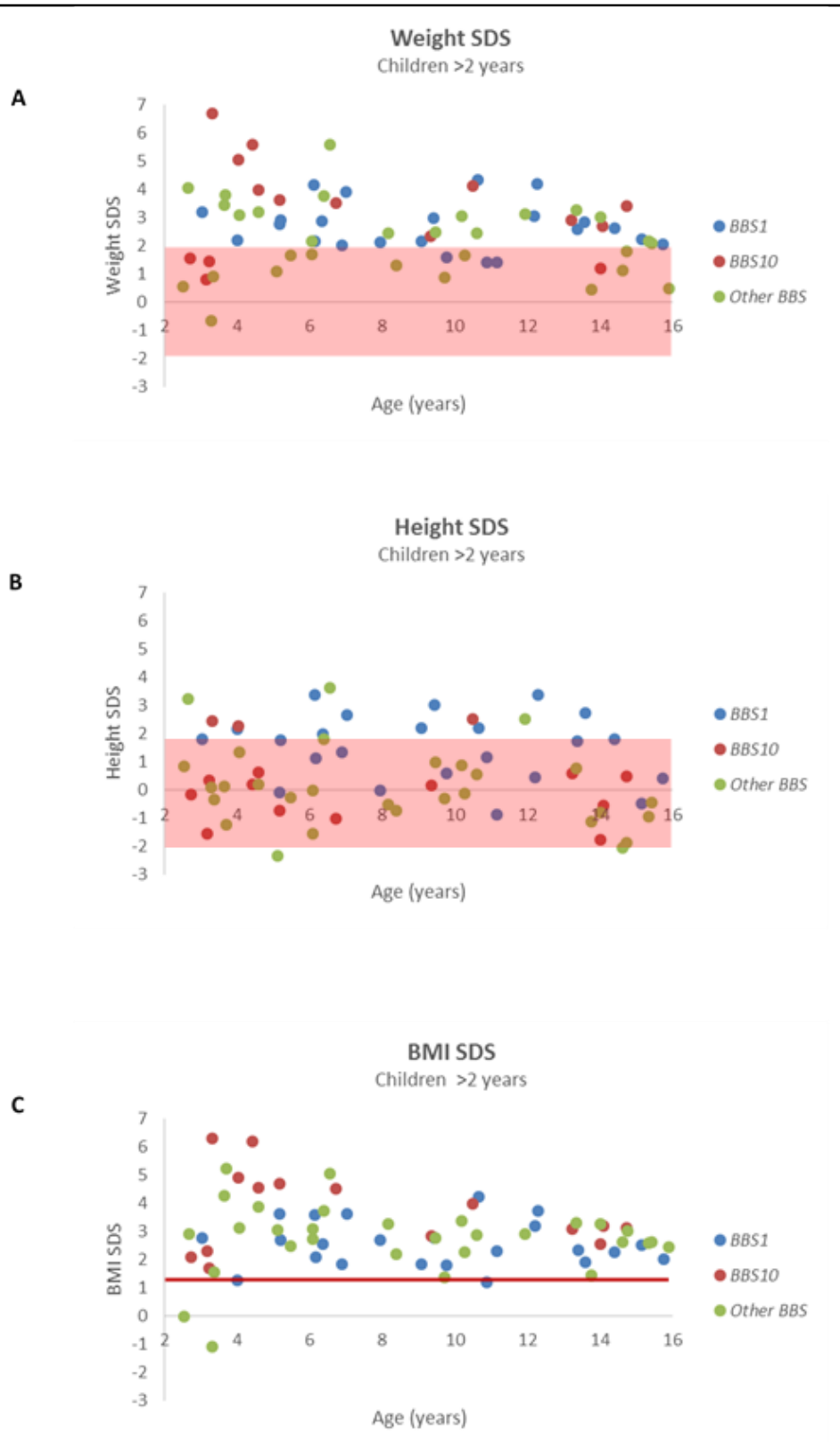


Figure 5.5 Height SDS and BMI-SDS in children over the age of 2 at first clinic attendance demonstrates signficant variability

A: Weight-SDS by age, the pink box represents the normal range. B: Height-SDS, the pink box represents the normal range. C: BMI-SDS, The red line represents the upper range for normal BMI-SDS (BMI-SDS of 1.33). There is evidence of a hypomorphic effect in *BBS1* mutations. Blue dots represent *BBS1* patients, red dots *BBS10* patients and green dots represent other genotypes.

5.2.1.3 *Paediatric patients have statistically significant reductions in BMI-SDS score on successive clinic attendances.*

The mean BMI-score at the first and second, third and fourth clinic attendance is within the severe obesity range (BMI-SDS > +2.67). BMI reduction with reference to the first clinic attendance improves with each visit and is statistically significant at each sequential visit. Table 5.1 outlines the mean BMI-SDS at each visit as well as the change in BMI-SDS at every visit with reference to the first visit.

Table 5.1 Change in BMI-SDS score on successive visits

Visit number	Mean BMI-SDS (SD)	Number	BMI-SDS change from first visit (SD)	T test comparison from first visit (P value)
1	2.92 (1.21)	72	N/A	N/A
2	2.77 (1.07)	58	-0.19 (0.56)	0.03
3	2.63 (1.12)	47	-0.49 (0.69)	0.0002
4	2.54 (1.10)	33	-0.57 (0.84)	0.002

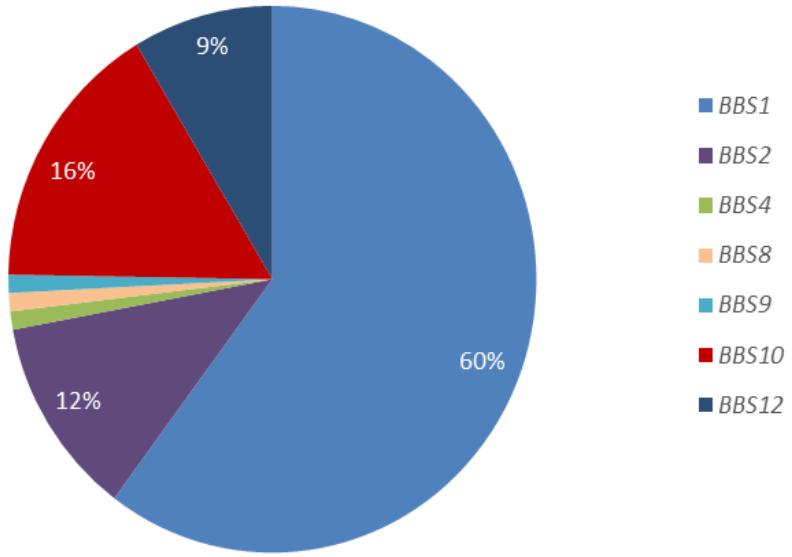


Figure 5.6 Distribution of genotypes in the adult BBS population

Mutations in *BBS1* predominate followed by mutations in *BBS10, BBS2* and *BBS12*.

5.2.2 Adults

One hundred adult patients had a recorded BMI at first visit to the national BBS clinics. Fifty-one patients were male and 49 female. Mean age at first attendance was 33 with an age distribution from 16 to 61. Mean time between hospital appointments was 1.6 years (SD: 0.8). Sixty patients had mutations in *BBS1*, 16 had mutations in *BBS10* and the remaining patients had mutations in other genes. The distribution of genotypes is shown in figure 5.6. Fifty-six had two missense mutations, 12 had a combination of missense and truncating mutations, 20 had two truncating mutations and the remainder had other mutation combinations.

5.2.2.1 Adult height for patients with Bardet-Biedl syndrome is shorter than the general population

Women with BBS had a mean adult height of 1.63m (SD: 0.09) and men with BBS had a mean adult height of 1.75m (SD: 0.09), both slightly shorter than the mean for women and men in the UK (1.65m and 1.78m respectively). Adults with *BBS1* mutations were taller on average than the general population and the BBS cohort as a whole. Women with mutations in *BBS1* had a mean height of 1.66m (SD:0.08) and men with mutations in *BBS1* have a mean adult height of 1.79 (SD:0.06). Adults with *BBS10* mutations were statistically significantly shorter than adults with mutations in *BBS1* (p=0.006) despite an equal males: female ratio. Of note, these were not ethnicity matched.

5.2.2.2 Patients with BBS10 mutations are more likely to be severely obese

To assess the prevalence of obesity among patients with BBS, BMI at first clinic attendance was charted. Weight was categorised according to BMI classification: normal BMI (18.5-25 kg/m^2), overweight (25-30 kg/m^2), obesity class I (30-35 kg/m^2), obesity class II (35-40 kg/m^2) and obesity class III (40+ kg/m^2)[196]. Figure 5.7 illustrates the distribution of BMI for adult participants in this study at first clinic attendance and figure 5.8 demonstrates the prevalence of BMI categories

according to genotype (*BBS1* versus *BBS10*) at first clinic attendance, demonstrating the higher propensity towards severe obesity in the *BBS10* group.

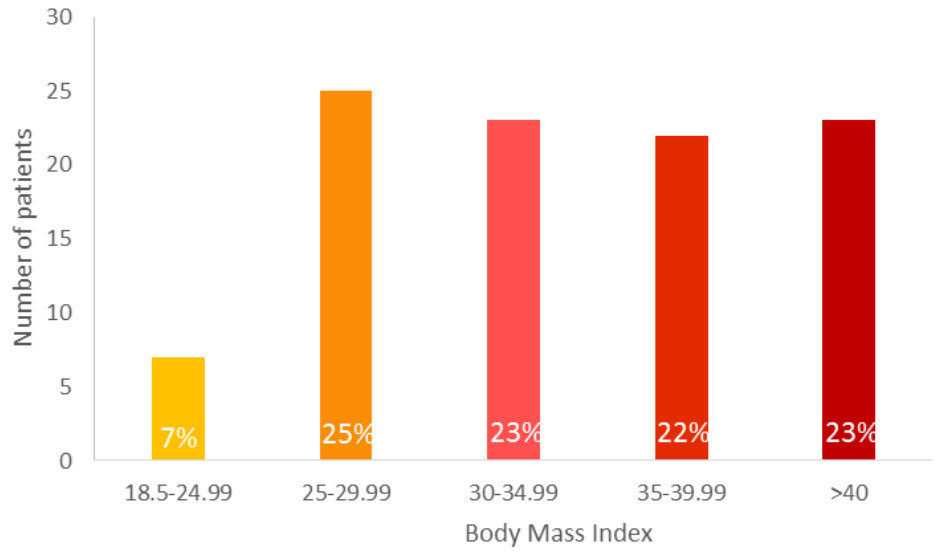


Figure 5.7 Distribution of BMI at first clinic attendance among patients with clinical and molecular confirmation of BBS.

Percentages denoted in each column. N=100. Only 7% of patients have a normal BMI. Sixty eight per cent of patients are classed as obese.

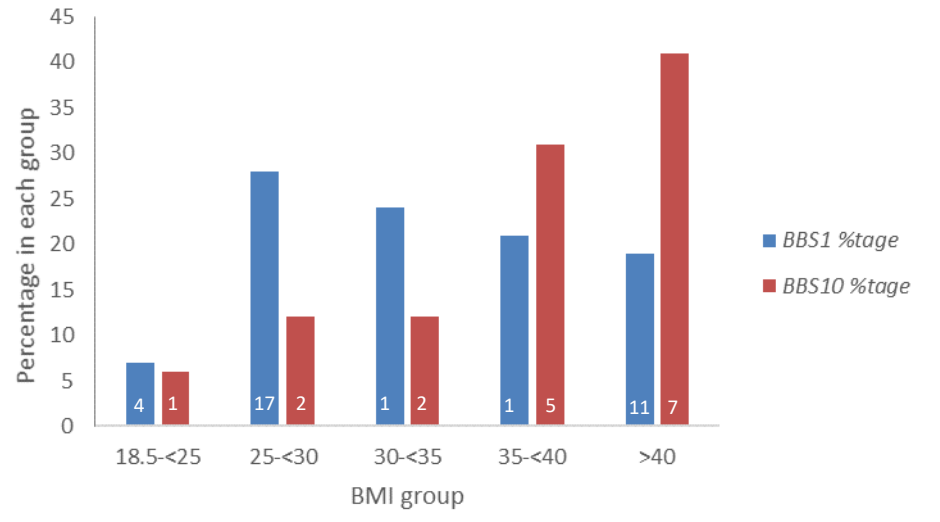


Figure 5.8 Patients with mutations in BBS10 are more likely to be severely obese

This figure demonstrates the percentage of patients in different BMI categories within each genotype. Absolute figures are given in each column.

5.2.2.3 *Obesity increases with age especially in patients with BBS10 mutations*

The variation in BMI at first clinic attendance is considerable. In keeping with the general population, there is an association between increasing age and higher BMI scores. For patients with mutations in *BBS10* this correlation is marked with a correlation coefficient of R=0.78 indicating a strong positive correlation between BMI and age.

Despite the higher percentage of *BBS10* patients being severely obese and the greater correlation between BMI and age, the difference in BMI was not statistically significant between patients with mutations in *BBS1* and *BBS10* (p=0.12). Given the correlation between weight and age this may be a reflection of the fact that *BBS1* patients are significant older than *BBS10* patients taking part in this study (p= 0.0002). Figure 5.9 demonstrates the correlation between BMI and age with colour coded genotypes.

5.2.2.4 *Attending BBS clinics is associated with improvements in BMI*

There was a small but consistent reduction in BMI with reference to first visits ranging from -0.1 to -0.3 on successive visits to the BBS clinics. Given that an increase in BMI is expected over time this is a positive result, despite not reaching statistical significance.

Table 5.2 demonstrates the mean BMI change on successive visits to the national BBS clinics with reference to the first clinic visit.

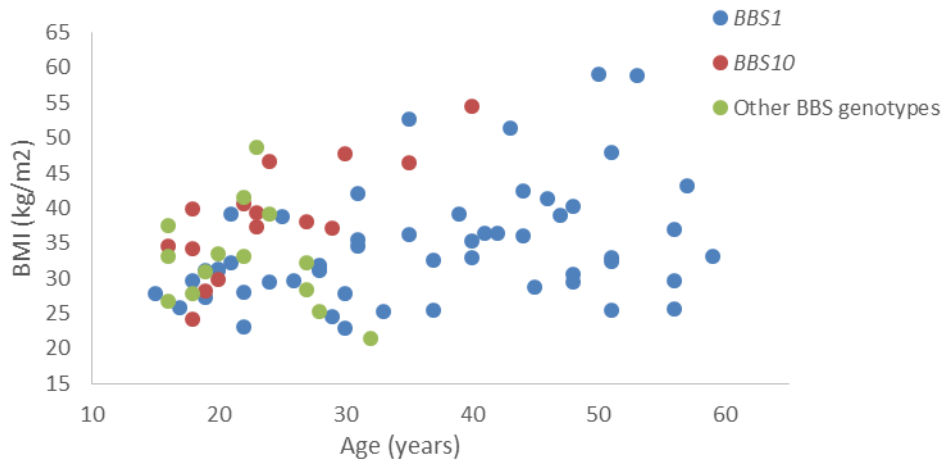


Figure 5.7 BMI according to age and genotype at first clinic attendance

All patients with *BBS1* mutations are denoted in blue, patients with *BBS10* mutations are in red and all other genotypes in green.

Table 5.2 BMI change with reference to first attendance at the national BBS clinic

Visit number	Mean BMI (SD)	Number	BMI change (SD)	P value
1	35.7 (8.1)	80	N/A	N/A
2	35.6 (8.2)	75	-0.3 (8.1)	0.85
3	35.5 (8.7)	62	-0.2 (3.2)	0.32
4	35.5 (7.7)	32	-0.3 (3.3)	0.12
5	35.4 (7.7)	20	-0.1 (3.4)	0.66
6	34.3 (5.2)	12	-0.3 (3.0)	0.30

5.3 Discussion

The obesity phenotype in BBS is poorly understood and no published data explores the natural history of this aspect of the phenotype despite the considerable risk of sequelae including cardiovascular morbidity, type 2 diabetes, renal disease and cancer.

The degree of obesity in each child is subject to a number of environmental factors. Examining the obesity levels at the first clinic attendance limits the effect of BBS specific dietetics input in order to get the best overview of the obesity phenotype and the variability within it.

Only 5% of children had a BMI-SDS within normal range and 83% were obese at the first clinic visit. In comparison, 7% of adults had a BMI within the normal range and only 65% were obese. This is a surprising finding given that BMI is expected to increase with age, and indicates a lower prevalence of obesity in the adult population than previously reported[3,6]. In both cohorts, patients with mutations in *BBS10* were more likely to be severely obese. One explanation for the discrepancy in incidence of obesity in the paediatric and adult cohort could be the difference in genotypic composition. There are considerably more adult patients with mutations in *BBS1* than in the paediatric population. Since patients with mutations in *BBS1* are less likely to be obese this may explain the discrepancy. The common *BBS1* p.Met390Arg mutation is primarily seen in Caucasian individuals and the difference in genotype composition may be a reflection of a difference in ethnicities presenting to the paediatric versus adult clinics.

There was considerable variability in weight-SDS and BMI-SDS in children at the first visit. This discrepancy is most marked in children below the age of six. It may be a result of a number of factors. Delayed walking and gross motor skills development is seen in many patients. Many of these patients may also not have been given a diagnosis yet and therefore not have had adequate medical advice and dietetic input to prevent accelerating obesity. The variability in weight indicators is most marked for patients with non-*BBS1* mutations and decreases with increasing age when most patients have a BMI-SDS between 2 and 3. This may be a reflection of the fact that most young people at this

stage will have had some degree of dietetics intervention regardless of whether they have had a diagnosis or not.

The adult cohort demonstrated an interesting pattern in the distribution of BMI at first clinic attendance according to genotype. As expected, there was an increase in BMI with increasing age. This increase was markedly steeper for patients with *BBS10* mutations than for other mutation types. Also, it was notable that the older patients all had mutations in *BBS1*. It is unclear why this may be the case. It is possible that patients with other genotypes struggle to attend the clinics as they are generally more severely affected with BBS with respect to visual function, cognition, renal disease and potentially sequelae relating to metabolic syndrome. It may also partly be a reflection of larger Caucasian population in the adult clinics.

BMI-SDS reduction with each sequential clinic was observed in the paediatric clinics with patients losing a statistically significant 0.19, 0.49 and 0.57 BMI-SDS score in comparison to baseline at each visit. Previous research in obese children and adolescents has shown that an annual BMI-SDS reduction of >0.25 improved insulin sensitivity, total cholesterol/high-density lipoprotein ratio and blood pressure. Greater BMI-SDS reduction was associated with greater cardiometabolic benefits[197,198]. This is particularly of interest in the context of a recent study which showed that increased risk of death from cardiovascular disease in adults has been found in subjects whose BMI had been greater than the 75th percentile as adolescents regardless of subsequent weight loss[199]. Results from the paediatric BBS clinics demonstrate that patients attending the clinics regularly over several years attain an impressive mean BMI-SDS reduction (-0.57 by the fourth visit). Ford *et al*[197] demonstrated that in non-syndromic obese children a BMI-SDS reduction of >0.5 significantly improves both body composition and metabolic risk

factors including a 30% reduction in triglycerides, a 15% reduction in Low-density lipoprotein-cholesterol and 45% reduction in CRP. Children with BBS attain an impressive BMI-SDS reduction given that they are genetically predisposed to obesity and are coping with many other aspects of the disease simultaneously.

Obesity in the adult population (BMI>30) was present in 68% of adults which is lower than expected based on previous estimates ranging between 72% and 92%[3,6]. BMI reduction on sequential clinic attendances was not as profound in the adult population as in the paediatric population, and the weight loss remained modest with reference to the baseline for each sequential visit (BMI-SDS change: -0.1 to-0.3). This may reflect the fact that weight loss is easier to attain whilst still growing in height and should be assessed in the context of the expectation that a net BMI increase is expected with increasing age. Therefore, this in itself is a clinically notable result albeit not statistically significant.

Individuals affected with BBS do not on average attain the same adult height as people who are not affected with BBS. However, many children appeared to have a height greater than +2 BMI-SDS, particularly children with mutations in *BBS1*. It is well documented that overweight and obese children are taller and reach their final height earlier than children of normal weight[200]. Interestingly, adults with *BBS1* mutations were significantly taller than adults with mutations in *BBS10* and attained similar height to the UK average male and female height supporting the theory that mutations in *BBS1* have a hypomorphic effect.

Limitations The primary limitation of this study pertains to the reliance on BMI and BMI-SDS scores as a proxy marker for obesity and, by extension, as markers of metabolic syndrome. Although BMI cut-offs to define obesity are based on well-defined indicators

of cardiometabolic morbidity and mortality, BMI cannot distinguish between lean mass and fat mass and does not give an indication of visceral fat. BMI has a high specificity (0.9) but a low sensitivity (0.5) for identifying obesity[201] and the relationship between BMI, obesity and cardiometabolic risk differs between ethnicities[201]. Dual Energy X-ray Absorptiometry (DEXA) scanning is a gold standard for determining body fat and identifying visceral fat, but this is not practical on a larger scale and would add considerable expense[201]. Considering these limitations, BMI is an adequate tool for assessing obesity in the BBS population.

This study was somewhat limited by taking place on two sites where there are minor differences in the service as well as demographic differences in the populations. Although patients attending both sites receive 18 monthly dietetics input, additional *ad hoc* telephone follow up is offered at the paediatric site. This is not available at the adult site and therefore there is a discrepancy in the service offered. It would be interesting to investigate whether patients who have attended the paediatric service continue to lose weight following transition to adult services or whether weight loss plateaus in keeping with other adult service users.

There is also a difference in the distribution of genotypes between the adult and paediatric populations. The reason for this remains unclear since both sites cover the same geographical area. It is possible that the older adults who are able to travel to the clinics are primarily individuals with mutations in *BBS1* because they are less severely affected, hence skewing the genotypic data.

Future directions Further work is required to understand the obesity phenotype in BBS. This work should include analysis of the effect of weight loss on cardiometabolic disease indicators and morbidity to assess for any differences in comparison to the general BMI-

matched population. Additionally the introduction of bioelectrical impedance analysis using body composition monitors (for example in the form of Tanita scales) would offer an opportunity for a more sophisticated understanding of adiposity. Liver ultrasonography would identify the proportion of patients affected or at risk of Non-Alcoholic Fatty Liver Disease (NAFLD) and add further to understanding of visceral obesity in this population.

Understanding how satiety affects obesity in this population would provide valuable insight into how much hunger contributes to obesity and therefore inform clinicians how best to manage the condition. Satiety scales have been used extensively in the PWS population[191,192] and also previously in the BBS population[184].

A number of therapies have been trialled in order to tackle obesity in the general population and some of these have potential application for BBS patients. Like patients with mutations in the MC4 receptor, BBS is thought to disable the appetite regulating MC4 pathway. A study by Iepsen *et al* demonstrated the effect of Liraglutide, A GLP-1 agonist on patients with MC4 Receptor mutations[180]. GLP-1 is a polypeptide secreted from L cells in the intestine in response to a meal intake and is known for its appetite-inhibiting and weight loss effects, but it is unknown which neurons GLP-1 acts on in the hypothalamus to induce these effects. Secher *et al* suggested that GLP-1 agonists may act on GABA neurons to inactivate orexigenic POMC/CART neurons[202]. Alternatively, GLP-1 might bypass the MC4 pathway by acting directly on paraventricular nucleus neurons in the hypothalamus[203]. The study by Iepsen *et al* suggests that it is possible to bypass the MC4 pathway and optimise other appetite regulating pathways thus partly restoring normal weight homeostasis[180].

MC4 receptor agonists such as Setmelanotide are another potential future avenue for BBS patients. Setmelanotide is currently entering phase III clinical trials and has shown significant potential inducing between 7 and 12% total body weight loss in four BBS patients as well as a significant reduction in hunger scores. Further trials will establish if this effect is consistently present across the BBS obesity population.

This study confirmed the hypothesis that patients with mutations in *BBS1* have a less severe obesity phenotype.

Understanding the pathways that predispose people at high risk due to monogenic disease may generate further understanding of obesity in other individuals who are genetically predisposed to obesity. This may also unveil signalling pathways for potential therapeutic intervention which could be applied to monogenic obesity as well as common obesity.

6 Can patients with Bardet-Biedl Syndrome be stratified according to cardiovascular risk?

"Do people die earlier as a result of BBS?"

6.1 Introduction

Patients, parents and carers of people with BBS often ask if it is a life-limiting condition. Very little is reported about mortality in BBS. Only one study by Riise (1996)[204] directly addresses the issue. Here, death certificates of 14 patients with BBS are reviewed to investigate cause of death. The primary cause of death was renal dysfunction in six patients, two were unknown and six patients died of cardiovascular disease. Causes of cardiovascular disease included myocardial infarction (n=3), embolism and thrombosis (n=2) and intracranial haemorrhage (=1). The median age of death was 46.6 years with an age range of 24 to 67 years of age. This study reported on patients born between 1914 and 1959 who died between 1953 and 1992. The incidence of death as a result of renal disease has hopefully changed significantly since this study was first published. Changes in medical practice including better detection, evaluation and treatment of CKD[205], as well as greater access for people with rare diseases to renal transplantations should have improved the prospects for people living with BBS and renal disease. It is striking that the remaining patients with known causes of death died as a result of cardiovascular disease, although this is unsurprising given the high level of obesity and Type 2 Diabetes Mellitus in this patient group.

Patient deaths in the UK BBS national clinics have not been reported on, and only very few patients attending the clinics have died. This is likely an ascertainment bias since few patients over the age of 60 have attended the clinics. The cause of death of patients who attended the clinics who have died include accidents, obstructive sleep apnoea and

deep vein thrombosis. No patient attending the national clinics has died as a result of renal failure.

Cardiovascular disease usually occurs as a result of atherosclerosis[206], although it is recognised that some BBS patients have structural cardiac defects[49,56] which may predispose to cardiac disease.

Cardiovascular disease is recognised as a chronic inflammatory disease of the vascular vessel wall. This results from the transendothelial movement of Apo-B lipoproteins including Very Low Density Lipoproteins (VLDL), Intermediate Density Lipoproteins (IDL) and Low Density Lipoproteins (LDL)[206] into the subendothelial tissue. The presence of lipoproteins in the subendothelial tissue leads to infiltration of macrophages and T cells[207]. Inflammation induced by obesity is likely to accelerate atherosclerosis[206]. The most commonly measured inflammatory marker in clinical practice, C-Reactive Protein (CRP), is a validated independent risk indicator of myocardial infarction, stroke, and vascular disease[208,209]. CRP attaches to the plasma membrane of damaged cells causing apoptosis through activation of the complement cascade[209].

In a study by Mujahid *et al*[55] comparing the endocrine profile of patients with BBS to unaffected age-, sex- and BMI matched controls, those affected with BBS were statistically significantly more like to have cardiovascular risk factors including: metabolic syndrome, raised triglycerides, higher risk of hypertension, raised fasting insulin, raised Homeostatic Model of Insulin Resistance (HOMA-IR).

Evidence from another form of genetic syndromic obesity, Prader Willi Syndrome (PWS), offers an interesting comparison as it has some phenotypic overlaps with BBS, including obesity and intellectual disability, and is often part of the differential before a diagnosis of BBS is reached. In this condition, obesity and cardiovascular risk are thought to be

associated through the inflammation induced by obesity, oxidative stress and alterations in the protective Apo-B lipoprotein HDL[210]. Ferretti et al[210] compared plasma lipid levels and CRP of 30 control subjects of normal weight to 15 individuals with PWS and 13 BMI matched controls. This study demonstrated increased inflammation, estimated by raised CRP, and oxidative stress in PWS and BMI- matched controls. However, compared to BMI-matched controls, PWS patients displayed lower activity of paraoxanase-1 (PON1), an enzyme involved in the HDL-derived antioxidant and anti-inflammatory effect, as well as marked changes in the physicochemical properties of HDL. Other studies have demonstrated that PWS patients are at increased cardiovascular risk reflected in raised CRP[211] and triglycerides[212].

Hedgeman *et al*[213] found that all-cause mortality was 11 times higher in the PWS population compared to the general population. Increased risks were also found for a number of cardiovascular events including myocardial infarction, deep vein thrombosis and pulmonary embolisms[213]. Another study of 312 PWS deaths revealed that cardiovascular disease was second only to respiratory failure as a cause of death[214]. A genotype-phenotype correlation was noted demonstrating that patients with maternal disomy 15 were more likely to die of cardiovascular causes than patients with a 15q deletion[214].

6.1.1 Rationale

The small cohort of people affected with BBS worldwide means that very little is known about cardiovascular morbidity in BBS. It is likely to be increased compared to the general population as well as BMI matched controls, due to the higher prevalence of cardiovascular risk factors. Pathogenic mutations leading to BBS are seen most

frequently in *BBS1* and *BBS10*. The aim of this study was to explore if it is possible to stratify people affected with BBS for cardiovascular risk according to genotype so that those at higher risk can be identified with a view to increasing surveillance and management if required thus reducing mortality.

6.2 Results

6.2.1 Genotype, age and gender distribution of participants

Seventy three patients were included in the study on the basis of a clinical diagnosis of BBS and molecular confirmation of mutations in *BBS1* or *BBS10*. Fifty two patients had mutations in *BBS1* and 21 harboured mutations in *BBS10*. The majority of patients were from different families except for nine pairs and two sets of three siblings.

Mutation type was classified according to predicted severity. The majority of patients had either two missense mutations, two truncating mutations or were heterozygous for missense/ truncating mutations. Four patients had splice site mutations (two in *BBS1* and two in *BBS10*) and were excluded from the study. Figure 6.1 demonstrates the mutation types in *BBS1* and *BBS10* in this study.

The mean age of the remaining sixty nine patients included in the study was 28.25 years (SD:14.41; range 0-59). The mean age (SD) of patients with *BBS1* mutations was 30.5 (15.6) years. Patients with mutations in *BBS10* were younger on average with a mean age (SD) of 22.32 (8.9) years. The difference in age was statistically significant on Student's T test (p=0.034). Patients with mutations in *BBS1* consisted of 24 (48%) females and 26 (52%) males. Twelve (63.2%) patients with mutations in *BBS10* were female and seven (36.8%) were male.

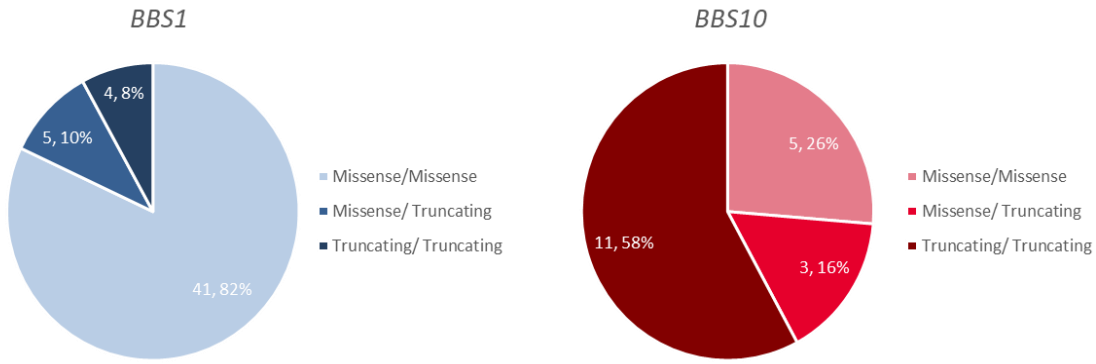


Figure 6.1 Mutation type distribution of participants with BBS1 and BBS10

Each pie chart demonstrates the absolute number and percentage of the genotype with each mutation type. Participant genotypes are skewed towards patients with missense mutations in *BBS1*. Adapted from Forsythe *et al*[4]

6.2.2 There is a high prevalence of clinical risk factors for cardiovascular disease in patients with BBS

Sixty five of the 69 patients included in this study had their blood pressure measured during clinic, and 23 were found to be hypertensive. Fifteen had a diagnosis of type 2 Diabetes Mellitus. Only five patients had the results of an echocardiogram documented in the notes. One of these patients had an atrioventricular defect, one had aortic valve stenosis, two had a ventricular septal defect and one had an innocent murmur. All patients with documented echocardiograms had two pathogenic variants in *BBS1*. Sixty seven patients in total had a full lipid profile; of these, 14 had hypercholesterolaemia.

Figure 6.2 demonstrates the distribution of clinical features in *BBS1* and *BBS10* as well as in the different mutation combinations.

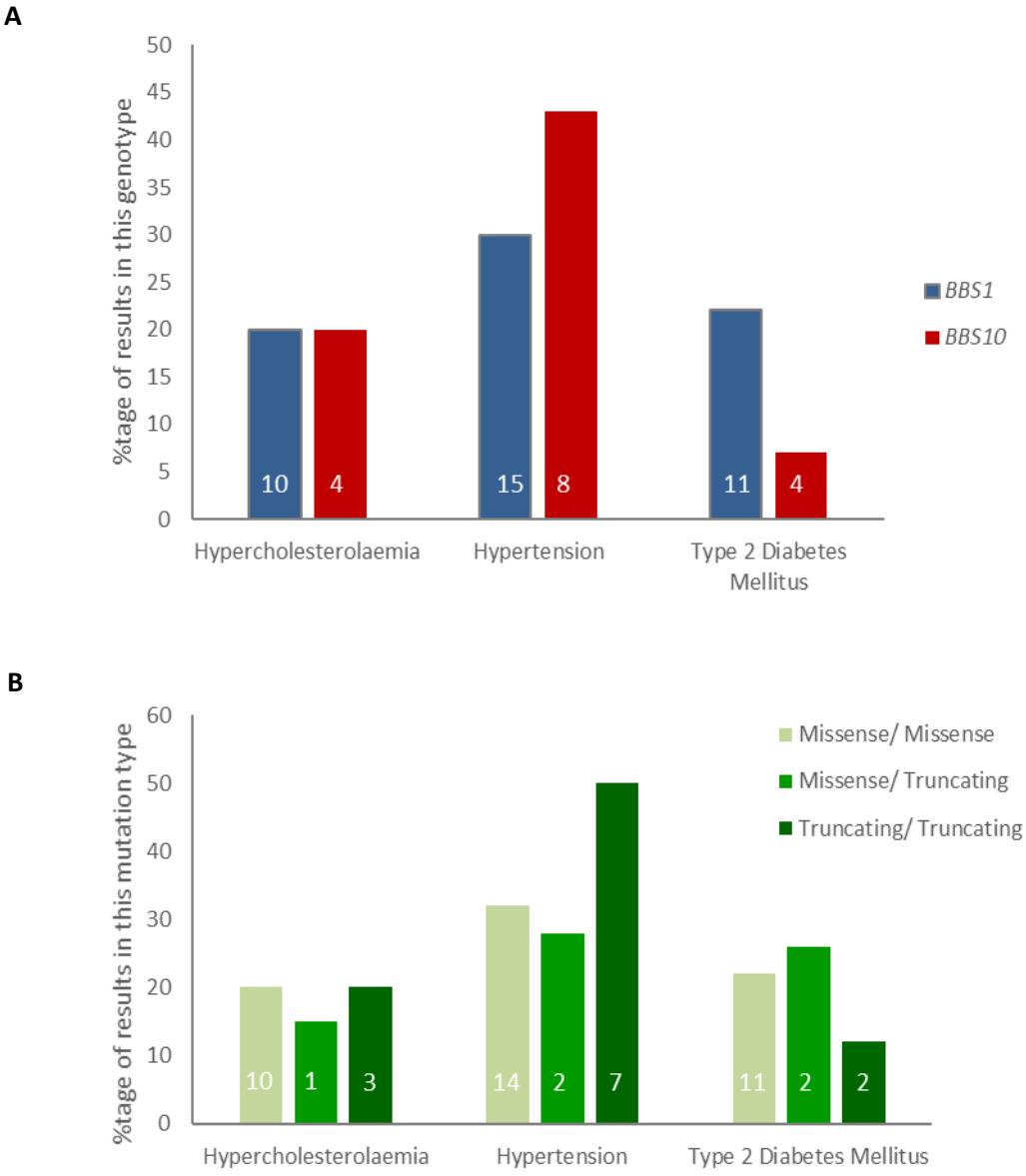


Figure 6.2 Clinical cardiovascular risk factors by (A) genotype and (B) mutation type

The prevalences are represented graphically by percentage of the total within each genotype/ mutation type to compensate for different population sizes. Absolute numbers are denoted in each column. Adapted from Forsythe *et al*[4]

6.2.3 Genotype and mutation type are associated with markers of cardiovascular risk including inflammatory markers, lipid profile and liver function.

All anthropomorphic and biochemical variables assessed in clinic were analysed for genotype- and mutation type –phenotype correlations by univariable analysis. On

comparing patients with mutations in *BBS1* and *BBS10,* there was a statistically significant difference in a number of clinical parameters including age, height, C-reactive protein (CRP), c-peptide, triglycerides, Potassium and albumin-creatinine ratio (table 6.1). Univariable comparison of the mutation types (two missense, two truncating and compound missense/ truncating) revealed a statistically significant differences in High Density Lipoprotein (HDL) and Gamma Glutamyl Transferase (GGT) (table 6.2)

Table 6.1 Univariable comparison of genotype-phenotype correlations.

	BBS1		***BBS10***		
	Mean	**(SD)**	**Mean**	**(SD)**	**p-value***
Anthropomorphic measurements					
Age at Clinic	30.5	-15.48	22.32	-8.95	**0.034**
Systolic blood pressure (mmHg)	122.89	-13.87	117.69	-17.42	0.411
Diastolic blood pressure (mmHg)	77.47	-11.08	78.27	-9.4	0.676
Height (cm)	163.92	-28.17	162.93	-9.31	**0.031**
Weight (kg)	93	-35.64	94.22	-31.48	0.934
BMI	32.69	-8.08	36.17	-8.16	0.125
Inflammatory markers					
White cell count (109L)	7.23	-1.85	8.28	-2.26	0.074
CRP (mg/L)	5.69	-2.74	9.53	-7.12	**0.040**
Platelets (109L)	241.82	-65.28	233.2	-51.04	0.649
Endocrine profile					
Cortisol (nmol/L)	382.22	-154.94	302.69	-128.17	0.094
T3 (µg/dL)	5.18	-0.51	4.65	-1.06	0.507
C peptide (ng/ml)	1295.88	-740.13	2333.3	-1501.27	**0.014**
Insulin (mmol/L)	135.19	-108.32	308.22	-462.62	0.082
HbA1c (%Hb)	5.75	-0.67	5.86	-0.98	0.935
Blood glucose (mmol/L)	5.34	-1.81	5.71	-2.39	0.563
Lipid Profile					
Cholesterol (mmol/L)	4.6	-0.79	4.65	-0.84	0.827
Triglycerides (mmol/L)	1.5	-0.73	1.98	-0.94	**0.049**
HDL cholesterol (mmol/L)	1.24	-0.23	1.14	-0.27	0.163
LDL cholesterol (mmol/L)	2.69	-0.81	2.62	-0.71	0.722
Renal profile					
Sodium (mmol/L)	141.98	-2.97	141.83	-2.85	0.863
Potassium (mmol/L)	4.16	-0.48	4.38	-0.42	**0.015**
Estimated GFR	88.48	-24.71	80.38	-33.49	0.371
Calcium (mmol/L)	2.2	-0.23	2.17	-0.28	1
Magnesium (mmol/L)	0.9	-0.08	0.88	-0.13	0.232
Phosphate (mmol/L)	1.15	-0.31	1.35	-0.63	0.213
Albumin/Creatinine ratio	7.1	-22.39	5.3	-11.23	**0.032**
Creatinine (µmol/L)	77.7442	30.501	103.56	108.318	1
Urea (mmol/L)	5.6913	3.1569	6.3125	3.11009	0.278
Liver profile					
Albumin (g/L)	47.12	-3.3	45.83	-2.55	0.146
Total bilirubin (µmol/L)	8.86	-6.7	8.94	-5.59	0.842
Alkaline Transaminase (IU/L)	36.59	-28.76	34.67	-28.36	0.698
Gamma Glutamyl Transferase (U/L)	34.19	-20.63	54.8	-38.94	0.375

*p-value obtained from ANOVA test or Mann Whitney U test. . Modified from Forsythe *et al*, 2015

Table 6.2 Univariable comparison of mutation type-phenotype correlations.

	Missense/ missense		Missense/ Truncating		Truncating/ truncating		
	Mean	**(SD)**	**Mean**	**(SD)**	**Mean**	**(SD)**	**p-value***
Anthropomorphic measurements							
Age at Clinic	30.52	-15.6	28.25	-10.99	21.27	-9.79	0.096
Systolic blood pressure (mmHg)	123.59	-13.46	116.5	-13.38	118.64	-19.05	0.211
Diastolic blood pressure (mmHg)	78.59	-11.2	73.5	-6.57	77.62	-10.87	0.493
Height (cm)	163.12	-27.92	167.43	-16.74	163.12	-9.67	0.278
Weight (kg)	95.33	-33.76	99.2	-36.02	84.56	-35.62	0.289
BMI	33.84	-7.88	34.29	-9.17	33.26	-9.21	0.959
Inflammatory markers							
White cell count (10^9L)	7.22	-1.72	8.41	-2.47	7.87	-2.41	0.269
CRP (mg/L)	5.94	-2.9	5	-1.07	10.25	-7.83	0.309
Platelets (10^9L)	235.74	-65.97	269.33	-71.98	235.08	-40.15	0.453
Endocrine profile							
Cortisol (nmol/L)	371.03	-164.4	325.43	-82.59	344.5	-149.39	0.870
T3 (µg/dL)	5.23	-0.49	4.7	-0.57	4.65	-1.06	0.455
C peptide (ng/ml)	1436.8	-849.7	1257.8	-402.61	2052.7	-1602.64	0.479
Insulin (mmol/L)	151.79	-110.4	121.4	-95.21	291.03	-491.4	0.848
HbA1c (%Hb)	5.82	-0.69	5.63	-0.83	5.74	-0.96	0.540
Blood glucose	5.36	-1.93	5.86	-2.59	5.47	-1.96	0.944
Lipid profile							
Cholesterol (mmol/L)	4.65	-0.75	4.38	-0.73	4.65	-0.98	0.669
Triglycerides (mmol/L)	1.5	-0.71	1.63	-1.13	1.99	-0.86	0.158
HDL cholesterol (mmol/L)	1.28	-0.25	1.09	-0.12	1.1	-0.23	**0.022**
LDL cholesterol (mmol/L)	2.7	-0.79	2.55	-0.73	2.65	-0.8	0.881
Renal profile							
Na (mmol/L)	141.42	-2.93	142.38	-2.5	143	-2.93	0.187
K (mmol/L)	4.17	-0.49	4.15	-0.4	4.39	-0.45	0.101
eGFR	88	-23.08	85.71	-38.21	81.91	-31.9	0.827
Calcium (mmol/L)	2.19	-0.25	2.1	-0.35	2.26	-0.06	0.388
Magnesium (mmol/L)	0.9	-0.08	0.88	-0.16	0.89	-0.12	0.652
Phosphate (mmol/L)	1.17	-0.31	1.11	-0.26	1.35	-0.68	0.743
Alb/Cr ratio	3.83	-7.99	23.48	-51.33	4.57	-10.85	0.421
Creatinine (µmol/L)	76.32	24.19	94.38	62.11	103.47	116.39	0.925
Urea (mmol/L)	5.43	2.67	8.43	6.01	5.62	1.27	0.486
Liver profile							
Albumin (g/L)	47	-3.27	47.5	-2.73	45.67	-2.89	0.293
Total bilirubin (µmol/L)	8.53	-6.53	10.38	-7.42	9	-5.53	0.796
Alkaline Transaminase (IU/L)	32.03	-19.11	47.88	-38.06	40.13	-40.76	0.367

Gamma Glutamyl Transferase (U/L)	29.21	-16.22	70.33	-10.02	62.75	-40.01	**0.027**
Alb/Cr ratio	3.83	-7.99	23.48	-51.33	4.57	-10.85	0.421

*p-value obtained from ANOVA test or Kruskal-Wallis test. Modified from Forsythe *et al*, 2015

Multivariable analysis of selected clinical parameters associated with cardiovascular risk was conducted controlling for confounding factors to assess for genotype- and mutation type- phenotype correlations. Tables 6.3 and 6.4 show the results of the multivariable analysis including confounding factors controlled for in the analysis.

Table 6.3 Multivariable comparisons of genotype-phenotype correlation. Only statistically significant results are included in this table.

	β Estimate	**95.0% CI**	**p-value***
CRP (mg/L)			
Genotype			
BBS1	Reference	-	-
BBS10	4.08	(0.90, 7.25)	0.013
Age	0.06	(-0.05, 0.18)	0.295
BMI	0.1	(-0.08, 0.29)	0.266
C peptide (ng/ml)			
Genotype			
BBS1	Reference	-	-
BBS10	942.94	(32.26, 1853.61)	0.043
BMI	-4.86	(-67.84, 58.12)	0.876
Blood glucose	92.08	(-71.24, 255.41)	0.258

*p-value obtained from linear regression model. Modified from Forsythe *et al*, 2015

Table 6.4 Multivariable comparison of mutation type-phenotype correlations. Only statistically significant results are included in this table.

	β Estimate	**95.0% CI**	**p-value***
CRP (mg/L)			
Mutation type			
Missense/missense	Reference	-	-
Missense/truncating	-0.65	(-4.42, 3.12)	0.729
Truncating/truncating	5.33	(1.99, 8.68)	0.002
Age	0.06	(-0.05, 0.17)	0.272
BMI	0.14	(-0.03, 0.31)	0.11
Triglycerides (mmol/L)			

Mutation type			
Missense/missense	Reference	-	-
Missense/truncating	0	(-0.67, 0.67)	0.996
Truncating/truncating	0.56	(0.01, 1.11)	0.048
Gender			
Female	Reference	-	-
Male	0.52	(0.07, 0.98)	0.026
BMI	0.03	(0.00, 0.06)	0.05
Age	-0.01	(-0.03, 0.01)	0.452
Gamma Glutamyl Transferase (U/L)			
Mutation type			
Missense/missense	Reference	-	-
Missense/truncating	44.22	(17.90, 70.54)	0.002
Truncating/truncating	29.32	(8.72, 49.91)	0.007
Gender			
Female	Reference	-	-
Male	17.94	(4.12, 31.76)	0.013
BMI	1.05	(0.15, ,1.95)	0.025
Age	0.24	(-0.28, 0.76)	0.349

*p-value obtained from linear regression model. Modified from Forsythe *et al*, 2015

6.2.3.1 C –reactive protein

Patients with mutations in *BBS10* or truncating mutations were statistically significantly more likely to have higher levels of CRP than patient with *BBS1* or homozygous missense mutations respectively (p=0.013 and p=0.002). White cell count, reflecting a state of infection, was not significantly different in comparison to the reference groups. Age, gender and body mass were included in the multivariable analysis as confounding factors.

6.2.3.2 C peptide

Patients with mutations in *BBS10* were significantly more likely to have higher C peptide levels on multivariable analysis than patients with mutations in BBS1 (p=0.043). BMI and blood glucose were included in the multivariable analysis as confounding factors.

Multivariable analysis of mutation type-phenotype correlations did not reveal a statistically significant difference.

6.2.3.3 Lipid profile

Patients with two truncating mutations were significantly more likely to have higher triglyceride levels than patients with two missense mutations on multivariable analysis (p=0.048). On multivariable regression analysis, triglyceride levels did not correlate significantly with genotype (p=0.09). Confounding factors incorporated in the analysis included age, gender and BMI.

6.2.3.4 Liver function

Patients with two truncating mutations had significantly higher GGT than patients with two missense or compound missense/ truncating mutations (p=0.007 and p=0.002 respectively). Liver function tests did not otherwise reveal a statistically significant difference. Confounding factors included in the multivariable analysis were gender, age and BMI.

6.3 Discussion

This study aimed to identify evidence of cardiovascular risk factors in the BBS population with a view to stratifying patients according to clinical need.

A high prevalence of clinical cardiovascular risk factors including hypertension, hypercholesterolaemia and type 2 Diabetes Mellitus are evident in patients with BBS. These findings are echoed in studies performed by other groups[55,188]. The high prevalence of hypertension in patients with mutations in *BBS10* and two truncating

mutations is particularly striking given that only one person in these groups is over the age of 31. This indicates that cardiovascular risk is raised at a young age in BBS and provides further support for the hypothesis that patients with *BBS10* mutations or two truncating mutations are particularly at risk. The young age of people with BBS participating in this study (mean age 28.25 years) and associated high prevalence of hypertension, hypercholesterolaemia and type 2 Diabetes Mellitus, suggests a high cardiovascular risk in this population which is likely to increase with age.

The study demonstrates that patients with mutations in *BBS10* or with two truncating mutations are more likely to have higher CRP levels than patents with mutations in *BBS1* or two missense mutations. Of note, there was no statistically significant difference in white cell count or BMI between the two groups. Given that CRP is raised in infection, inflammation and obesity[215] this points to a possible independent effect in these overlapping patient groups. Several lines of investigation suggest a link between CRP and leptin indicating a relationship, and also that they are valid independent biomarkers of cardiovascular risk[216]. Feuillan et *al*[188] demonstrated that BBS is associated with hyperleptinaemia independently from co-morbid obesity. These results are supported by data from *Bbs2*-/-, *Bbs4*-/- and *Bbs6*-/- mouse models[217]. Further evidence for the link between leptin and CRP is provided by Zhang et al, who demonstrated that variations in the Leptin Receptor (LEPR) are associated with CRP levels as well as fibrinogen, another inflammatory marker.

Patients with mutations in *BBS10* have statistically significantly higher levels of C peptide than patients with mutations in *BBS1*. When insulin is synthesised, pro-insulin is cleaved into insulin and Connecting Peptide (C-Peptide). Although primarily used as a marker of insulin resistance, C-peptide is increasingly being recognised as a powerful biomarker of

both cardiovascular mortality[218] as well as all-cause mortality- in particular in colorectal cancer[219]. These findings suggest that patients with mutations in *BBS10* are at higher risk of cardiovascular death and all-cause mortality than patients with mutations in *BBS1*.

Patients with two truncating mutations are more likely to have raised triglycerides than patients with other mutation types indicating an increased cardiovascular risk in this group.

A statistically significant increase in GGT was demonstrated in patients with two truncating mutations and in those with compound missense/ truncating mutations. GGT is classically associated with chronic liver disease but is also an independent biomarker of cardiovascular risk as well as all-cause mortality[220].

Life expectancy and co-morbidities beyond those that relate directly to the clinical features of BBS have remained somewhat unexplored. The paper by Riise[204] highlighted the risks associated with renal disease as well as cardiovascular disease. Extensive efforts and resources are allocated in primary care to the prevention and management of cardiovascular risk factors in the general population, and the same should apply to high –need groups such as BBS patients. This study demonstrates not only that patients with BBS have indicators of significant cardiovascular risk at a young age, but also specifically that patients with mutations in *BBS10* and two truncating mutations are particularly at risk of cardiovascular morbidity and mortality. This calls for greater awareness and aggressive management of hypertension, hypercholesterolaemia and renal dysfunction in this high risk population.

Limitations. This study was limited by the timing of data collection for this study. At the time this study was conducted, the national BBS clinical services had only been in

operation for three years and hence the total number of patients is lower than that for other studies in this thesis. Furthermore, the data were collected before the BBS gene panel was introduced into clinical practice. Since molecular confirmation was a pre-requisite for inclusion into the study there is an ascertainment bias towards patients with the common *BBS1* p.Met390Arg and *BBS10* p.Cys91Leufs*5 mutations. This also means that it was not possible in this study to include mutation type as a confounding factor on multiple regression analysis, since very few patients with *BBS1* mutations were found to have truncating mutations and very few patients with *BBS10* mutations were found to have missense mutations. It remains to be seen if the more severe cardiovascular phenotype relates to the mutated gene, the mutation severity, or most likely, a combination of the two.

The young average age and the statistically significant age difference between participants with *BBS1* versus *BBS10* mutations is likely to limit the results as cardiovascular disease is likely to present at a higher frequency with increasing age.

Another limitation of collecting data through a national clinic is the limited access to past medical records, as a result, not all health data could be captured in this study. It is possible, for example, that more than five of the 69 participating patients have had an echocardiogram, which would provide further insight into the prevalence of structural and acquired cardiovascular defects. The prevalence of structural defects found in four of the five patients with evidence of echocardiography is likely to represent an ascertainment bias. Likewise, it would be helpful to see evidence of a liver ultrasound to ascertain if raised GGT was seen in association with fatty liver disease or present in isolation.

Multivariable regression analysis is used to assess the relationship between a dependent variable and several independent variables. The primary advantage of this type of analysis is that it allows for interrogation of the relative influence of a number of different variables[221]. Another advantage is that it facilitates the identification of outliers that could skew data interpretation[222].

A disadvantage of using a multiple regression model is the requirement for large data sets in order to avoid false conclusions on causation[223]. Another disadvantage is that the probability of incorrectly rejecting a true null hypothesis increases in parallel with the number of tested hypotheses in each analysis[221].

It has been argued that it is prudent never to correct for multiple comparisons, but instead report all individual P values and confidence intervals whilst clarifying that no statistical correction was made for multiple comparisons[224]. Alternatively, there are a number of commonly applied methods for adjustment. These include Bonferroni adjustment, which attempts to control family-wise error rate (the possibility of rejecting at least one null hypothesis)[221]. Other less conservative methods include Holm, Hommel and Hochberg adjustment[221].

A common rule of thumb states that at least 10 observations are required per independent variable examined as part of the multivariable regression analysis[222]. A total of 69 observations were available for this study, and each analysis contained four or five observations. Although it could be argued that the regression model used here is slightly overfitted, the results of the multivariable regression are consistent with the univariable results and do not appear to be overinflated.

Future work Future developments should focus on gathered data on a larger group of patients to ascertain if the findings identified in this study can be reproduced. This study provides insight into the cardiovascular risk factors of people living with BBS and sufficient evidence for heightened awareness of a likely increased risk of cardiovascular morbidity in people with mutations in *BBS10* and two truncating mutations. A bigger study, including more older individuals, could more precisely delineate the risks associated with different genotypes including other less common genotypes such as *BBS2* and *BBS12*. Charting the co-occurence of hyperleptinaemia and CRP in a future study would provide further insight into the link between these two biomarkers in the context of BBS.

A multi-national study could potentially lead to validated risk stratified clinic guidelines and aim to provide early, risk-reducing intervention, thus reducing overall mortality in this high risk patient group.

7 Do Bardet-Biedl Syndrome patient primary cell cultures have a phenotype that can be harnessed as a marker for therapeutic intervention?

"Are therapies on the horizon for BBS?"

7.1 Introduction

The development of novel therapies requires not only a comprehensive understanding of the clinical phenotype but also, ideally, of the aberrant cellular processes that underpin it.

In this chapter, I investigate whether three potential phenotypes, which have been documented in animal models and commercial cell lines are also present in primary human cell cultures: i. Decreased ciliation and cilium length ii. Increased cell proliferation iii. Aberrant expression of cilia specific genes.

7.1.1 Ciliation and cilium length in Bardet-Biedl syndrome

Given the pivotal role played by BBS proteins in cilia assembly and maintenance it is unsurprising that previous research has indicated that BBS deficient cells grow fewer and shorter cilia. Hernandez-Hernandez *et al*[225] investigated *bbs4*- and *bbs6*- mouse renal medullary cells and found that cilia were shorter, less abundant and associated with a disorganised cytoskeleton. Veleri *et al* also demonstrate reduced number and length of cilia in *bbs9* morpholino knockdown zebrafish[226]. Other groups have identified a more complex cellular phenotype. Mokrzan et al[227] and found that primary renal tubular cells from wild type and *bbs4*- mice had different patterns in developing cilia length- at seven days in culture *bbs4*- cells had shorter cilia and at 10 days longer cilia than control cells. These results were recapitulated *in vivo* in kidneys extracted from 3

to 4 month old *bbs4*+/+ and *bbs4*-/- mice. *bbs4*-/- renal tubular cells consistently grew slower than *bbs4*+/+ renal tubular cells.

No previously published work has investigated the ciliary phenotype in BBS patient samples. However, work by Srivastava *et al*[74] and Shimada *et al*[228] on patient samples from patients with mutations in the ciliary protein *CEP290* (*BBS14*) in patients with non-BBS phenotypes revealed evidence of a highly variable cellular phenotype. *CEP290* is associated not only with BBS, but also with a host of other ciliopathies including Joubert Syndrome (JS), Senior-Loken syndrome (SLS), LCA and Meckel Syndrome (MKS). Srivastava et al[74] demonstrated significantly longer cilia and poorer ciliation rates in comparison toalso UREC control samples derived from a patient with JS who had a renal phenotype. Surprisingly, the ciliary phenotype was restored to normal when patient cells were cultured with 20µM Purmorphamine, a Shh pathway agonist. The purported mechanism of action of Purmorphamine in partially rescuing the phenotype was hypothesised to relate to changes in the cell cycle and documented reductions in *CDK5* expression suggesting a convergence of the hedgehog and cell cycle pathways[74]. In contrast, URECs from another patient with *CEP290* mutations and clinical features consistent with LCA and no renal dysplasia did not reveal any abnormality in cilia length or ciliation[74]. Shimada *et al*[228] showed that fibroblasts from a patient with *CEP290*-JS demonstrated reduced cilia and a broader distribution of cilia length compared to controls. In contrast, fibroblasts from two LCA-CEP290 patients, where no renal phenotype is present, were not significantly different in ciliation or cilia length compared to controls[228].

The normal length and morphology of the cilium is dependent on the type of cell (see table 7.1). Although the cilium length varies between cell types, evidence suggests that

any variation from the normal range of a particular cell type is indicative of pathology[229]. The balance point model suggests that the intersection between assembly and disassembly rate is the length setting point[229]. It has been suggested that a potential cilia length sensing mechanism exists involving the CEP290 protein[230]. CEP290 is located in the transition zone and appears to regulate levels of Intraflagellar Transport (IFT) complexes and entry into the cilium[230].

Table 7.1 Cilia length varies between cell types

Cell type	***Cilia length***	***Reference***
Vascular endothelial cells	1-5 µm	Van der Heiden *et al*, 2008[231]
Kidney epithelial cells	5-6 µm	Besschetnova *et al*, 2010[232]
Neurons	4-9 µm	Broekhuis *et al*, 2013 and Myoshi *et al,* 2014[229,233]
Osteoblasts	3-4 µm	Qiu *et al,* 2012, Delaine-Smith, *et al* 2014[234,235]
Chondrocytes	2 µm	Wann *et al,* 2012[236]
Fibroblasts	3-7 µm	Shimada *et al*, 2017[228]

Adapted from Dummer *et al*, 2016 [230]

Several mechanisms exist for variation of cilium length: i. Alterations to the microtubular axoneme, ii. Modification of the IFT machinery, and iii. Regulation of signal transduction pathways.

Generation and maintenance of the ciliary axoneme plays a crucial role in determining ciliogenesis and cilia length. The microtubular axoneme is constructed from tubulin derived from the cell body tubulin pool[229]. Experiments have shown that tubulin is inserted at the distal axoneme and that turnover progresses slowly at a rate of approximately 5% per hour[237]. Post-translational modification of tubulin including acetylation, detyrosination, polyglutamylation and polyglycolation is abundant in

axonemal tubulin and is important for cilia stability, ciliogenesis and cilia length[229]. BBIP10, a component of the BBSome is required for microtubule acetylation[237].

Given the pivotal role that IFT plays in regulating cilium length, any disruption can have an adverse effect on length[232]. During primary cilia elongation, anterograde IFT velocity is increased while retrograde IFT velocity remains the same[229]. Although IFT disruption may result in shortened cilia such as in Jeune's Asphyxiating Thoracic Dysplasia (JATD)[238], it may also result in longer cilia depending on the point at which the equilibrium of anterograde and retrograde transport is disturbed[238]. It has been suggested that BBS proteins function as a glue that holds the IFT complex together, and that disruption results in IFT aberrations and ciliary length defects[239,240].

Mutations in genes coding for proteins that localise to the transition zone also appear to have an effect on cilium length and ciliogenesis[229]. These proteins include those linked to MKS and Nephronophthisis. In chlamydomonas *CEP290* knockout models there are aberrations in the protein composition and structure of the flagellum, including insufficient Y links and accumulation of IFT-B and BBS4 proteins[241]. It seems likely that transition zone and basal body proteins play an important role in modifying IFT hence influencing cilia length[229].

A number of signalling pathways have been implicated in regulating cilia length and ciliation. cAMP is thought to play a role in regulation of cilium length on the basis that inhibiting adenylate cyclase leads to cilia length increase [242,243]. Downstream effectors of cAMP such as MAP kinase, Proteinase Kinase A and cyclic nucleotide-gated channels also have an effect on cilia length[232,244]. Other signalling molecules that affect cilium length and ciliogenesis were identified through an RNA interference screen and include regulators of actin and endocytic recycling[245]. Regulation of the mTOR pathway also

affects cilium length. *Tsc1* knockout mice and *TSc1a* knockdown zebrafish possess elongated cilia[246,247], and identified that a decrease in cilia length in zebrafish could be rescued on treatment with mTOR inhibitor rapamycin[248]. Further signalling pathways that have been implicated in cilia length and ciliogenesis include fibroblast-growth factor, Notch signalling, Never In Mitosis A (NIMA)- related kinases and the RCK-kinases[229].

7.1.1.1 Environmental factors can influence cilia length and ciliogenesis

External factors play a significant part in determining ciliary length and ciliation. Cells grown in a monolayer respond to confluence, medium components, and time spent in serum starved medium[249]. However, other external factors may also play a role and remain somewhat unelucidated[249].

One group of investigators demonstrated that renal injury may induce cilia elongation through a signalling cascade linked to hypoxia[250]. Evidence from renal epithelial cell primary cilia following exposure to renal tubular necrosis demonstrates prolonged cilia[251,252]. MDCK (Madin Darby Canine Kidney) cells exposed to simulated hypoxia through stabilisation of Hypoxia-Inducible Factor-1α (HIF1α) also demonstrate elongated cilia[250]. It has been hypothesised that the elongated cilia improve the sensory function of the cilium which may be crucial during the repair phase[249].

Exposure to lithium elongates primary cilia in the mouse brain and cultured cilia including fibroblast-like synoviocytes[243]. This is thought to occur through inhibition of Adenylate Cyclase-3 (AC3) and cAMP[249].

Deflection of cilia in MEK and IMCD3 cells has been shown to decrease intracellular cAMP levels and shortening cilia as a result and a subsequent decrease sensitivity to the

mechanical stress of fluid flow. This negative feedback loop has can be disrupted by knockdown of PC1 and PC2[253,254].

7.1.2 Role of the cilium and BBS proteins in proliferation and the cell cycle

The role of ciliary proteins known collectively as the ciliome in the cell cycle is only beginning to come to light[255]. Cilia typically form during G0/ G1 and disassemble as cells re-enter the cell cycle[256]. Entry into G0/ G1 induces differentiation of the centrosome into a basal body by the addition of ciliary rootlets, transition fibres and basal feet[256]. The presence of cilia prevents entrance into the cell cycle through sequestering the basal body[256]. This is due to the simultaneous requirement for basal bodies/ centrioles in ciliogenesis and the cell cycle[255,257]. When cilia are resorbed, the centrioles are used to form microtubule organising centres for the mitotic spindle[257]. Cilia disassembly occurs at the G1/S progression and immediately before M phase entry[257]. When cells are induced to form longer cilia it delays cell cycle re-entry from G1 to S phase[255].

Unregulated cell cycle progression can prevent cilia formation such as is the case in cancer cells[258], or may result in cystogenesis in susceptible organs for example the kidney[259].

A number of cilia proteins contribute to regulating the cell cycle (figure 7.1). Axoneme disassembly proteins including dynein sub-unit interactor and mother centriole protein NDE1 suppresses ciliary formation and cell cycle entry[255] whereas when cytoplasmic dynein protein TCTEX1 is phosphorylated it induces ciliary resorption and promotes S phase entry[260]. INPP5E, a phosphatase localising primarily to the ciliary axoneme and associated with Joubert syndrome has been found to accelerate ciliary disassembly[261]. Aurora A is a centrosomal kinase that regulates mitotic entry via spindle organisation

and is also required for cilia disassembly in hTERT-RPE cell[262]. IFT88 is required for proper spindle orientation in mice and mutations in IFT88 have been shown to result in overproliferative kidney cysts in the mouse [263,264]. Never In Mitosis-related kinases are involved in the cell cycle and are also thought to be involved in regulating both ciliogenesis and the cell cycle[265]. NEK1 has been found to not only play a crucial role in DNA repair and promoting progression to M phase but also plays a role in centrosome stability[265].

The role of BBS proteins in the cell cycle is less well understood. *BBS4* has been identified as a significant contributor to the cell cycle. HeLa cells depleted of *BBS4* fail to divide and individual *BBS4* depleted cells often have 2 or more nuclei of variable sizes. BBS4 is a pericentriolar protein with a key role in recruiting centriolar satellites, protein aggregates involved in centrosome maintenance and ciliogenesis. BBS4 appears to form a complex with PCM1 and loss of BBS4 results in PCM1 dispersal in the cytosol, microtubule disorganisation and alteration of the cell cycle with a higher proportion of cells undergoing apoptosis[266]. Furthermore, *BBS4* silenced 3T3F442A pre-adipocytes demonstrate accelerated cell division and aberrant differentiation[96]. When BBS4 was re-introduced proliferation reverted to wild type[267]. It has been suggested that *BBS6* may also play a role in cell division as it continues to be associated with centrosomes during the cell cycle[268].

A role for other BBS proteins in the cell cycle has not yet been delineated, this may be a product of an as yet unknown function. Alternatively, since these proteins act in concert, dysfunction or absence of one BBS protein may adversely affect the function of BBS4.

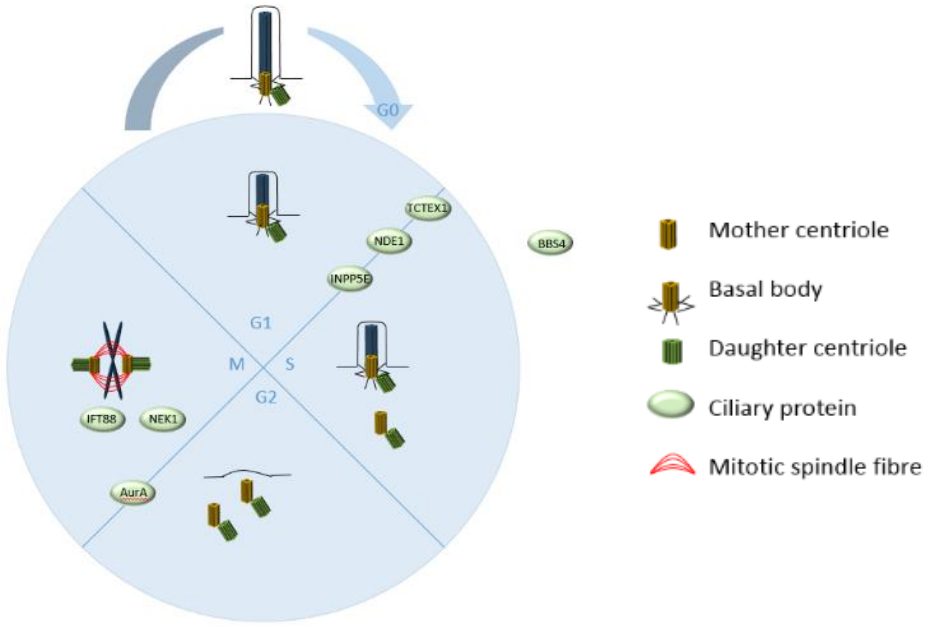


Figure 7.1 Cilia and the cell cycle

Cilia assembly and disassembly throughout the cell cycle alongside known ciliary proteins with dual function in cell cycle progression. In most cells the cilium forms during G1. During S phase the centrioles and DNA replicate. Cilia disassemble aduring G2/M and reassemble at G1. The ciliation rate and cilia length peaks when cells are quiescent (G0).

7.1.3 Cilia gene expression patterns in BBS patient samples

Although aberration of several signalling pathways hascilia been implicated in ciliopathies, our understanding of which pathways are specifically implicated in the pathogenesis of the pleiotropic effects observed in BBS remain only partly understood. Identifying aberrations in primary cilia gene expression in samples from patients with BBS could identify target pathways for future therapeutic developments, provide markers of therapeutic success, as well as potential biomarkers of disease severity.

The Hedgehog pathway is one of the molecular pathways most strongly associated with primary cilia[11]. Aberrations in this pathway have been linked to both limb bud development[11] and renal fibrosis[269] which may account for the polydactyly and (in part) chronic kidney disease in BBS[3]. The hedgehog receptor Patched1 (PTCH1) localises to the cilium[11]. On hedgehog binding, downstream transmembrane protein Smoothened

(SMO) accumulates within the cilium where it converts transcriptional factors Glioma-associated oncogenes GLI1, GLI2 and GLI3 from repressors to activators[11]. These proteins have a dual role in regulating signalling in the cilium, activating transcription in the nucleus and participating in cell proliferation, survival and metastasis[11,255].

The Wnt pathway is another primary cilia mediated pathway. It is involved in planar cell polarity, cell migration, skeletal development and organogenesis and has been linked to renal cystogenesis in BBS[3]. Wnt binds to membrane bound protein Frizzled (FZD) which activates either the canonical or non-canonical Wnt pathway[270]. The cilium/ basal body may function as a regulator of canonical/ non-canonical Wnt signalling. In canonical Wnt signalling the presence of β-catenin is high due to the inhibition of the β-catenin destruction complex[16]. The complex is composed of Axin (AXN), adenomatous polyposis coli (APC), casein kinase 1 (CK1), and GSK-3β[270]. In the absence of Wnt phosphorylation of β-catenin by CK1 and GSK-3β targets β-catenin for degradation[270]. In the presence of Wnt, Dishevelled (DSH) is inactivated and β-catenin levels rise and translocate to the nucleus where it acts as a transcriptional coactivator[255].

The non-canonical Wnt signalling pathway is less well characterised, and is primarily involved in cell migration and planar cell polarity[3]. INV is thought to deliver the signal for the non-canonical Wnt pathway[255].

Several components of the Wnt pathway can bind to Polycystic Kidney Disease -1 (PKD1) which can mediate the effect of other Wnts both in the kidney and elsewhere making this a candidate pathway for the molecular pathogenesis of renal disease in BBS[270].

Mammalian Target of Rapamycin (mTOR) is another signalling pathway that has been implicated in renal cystogenesis in the ciliopathies. mTOR is a serine/threonine protein kinase involved in the cell cycle, proliferation and growth. This pathway has primarily

been studied in the context of renal development[271]. The proposed pathogenesis is that normally functioning cilia on renal cells deflect in response to the flow of urine which activates Tumour suppressor kinase Liver Kinase B1 (LKB1) and facilitates phosphorylation of AMP-dependent protein kinase (AMPK)[270]. This allows AMPK to activate the TSC1-TSC2 complex which stimulates Ras-Homologue-Enriched-in-Brain (RHEB) which in turn inhibits mammalian-Target-Of-Rapamycin-Complex-1 (mTORC1)[257]. PC1 may also inhibit mTORC1 thus facilitating cross talk between the mTOR and Wnt signalling pathways[270]. TSC1 and TSC2 have also been reported to regulate SMO dependent HH signalling in mouse embryonic fibroblasts[272].

Notch signalling is an important pathway in determining left-right asymmetry, regulating cilium length and enhancing the response of neural progenitors to Shh[273]. Other functions include maintenance of tissue growth and development and neurogenesis and depends on the primary cilium for signal transduction[270]. Zebrafish with *bbs1* and *bbs4* defects accumulate Notch receptors in the late endosomes thus inhibiting receptor degradation[274].

Transforming growth factor β (TGF-β) signalling plays an important role in bone development and maintenance by regulating cell cycle progression and migration. A TGF-β1/Bone-Marrow-Protein (BMP) complex is released in the event of bony injury[275]. TGF-β receptors localise to the ciliary tip and endocytic vesicles at the ciliary base implicating the primary cilium in this signalling pathway[270].

Platelet-derived growth factor (PDGFR) signalling mediates signalling through the primary cilium. This receptor tyrosine kinase plays an important role in cell cycle progression, proliferation, migration and embryonic development[276]. Upon ligand binding the Mitogen-activated protein kinase kinase /Extracellular signal–Regulated

Kinases (MAPK-ERK) pathways are triggered, and aberrations in this pathway are associated with cancer, inflammation and fibrosis[270].

7.1.4 Rationale

Published evidence examining the cellular phenotype in Bardet-Biedl syndrome (BBS) refers almost exclusively to work done in commercial cell lines and animal models. Although this has given some preliminary insight, a consistent phenotype must be identified in primary human cell cultures in order to deliver robust outcome measures for the purpose of risk stratification and therapeutic developments.

7.2 Results

7.2.1 Cell cultures

Fifty four primary human fibroblast cell cultures were established from BBS patients and two cell cultures (CTRL1 and CTRL2) from controls. A further six cell cultures were acquired from the Medical Research Council Centre for Neuromuscular Disease Biobank, UCL Great Ormond Street Institute of Child Health. Appendix 1 provides an overview of all the patient fibroblasts samples collected and cultured for this study. Tables 7.2 and 7.3 demonstrate the samples used for the experiments reported here, highlighting basic participant data as well as which samples were used to assess, ciliation and cilia length, proliferation and gene expression.

Nine human urine derived epithelial cell cultures were established from BBS patients and 10 controls. Tables 7.4 and 7.5 demonstrate all samples successfully cultured for the purposes of this study and highlights basic participant data as well as which samples were used to assess ciliation and cilia length, proliferation and gene expression. The renal phenotype for each patient is also recorded demonstrating the renal phenotypic variability. Of interest, six of the nine samples that proliferated and therefore could be used for this study are from patients with known renal structural abnormalities.

Table 7.2 BBS human fibroblast primary cell cultures

Code	*Gene*	*Mutations*	*Age*	*Gender*	*Used for ciliation and cilia length Assays*	*Used for proliferation assay*	*Used for gene expression array*
RT2	*BBS2*	c.565C>T/p.Arg189*/ c.565C>T/p.Arg189*	20	M	X		
CO1	*BBS10*	c.55G>T/p.Glu19*/ c.590A>G/p.Tyr197Cys	26	M		X	
CO6	*BBS1*	c.1169T>G/p.Met390Arg/ c.1169T>G/p.Met390Arg	27	F		X	X
GST04	*BBS1*	c.1169T>G/p.Met390Arg/ c.1169T>G/p.Met390Arg	54	F		X	
GST05	*BBS1*	c.1169T>G/p.Met390Arg/ c.1169T>G/p.Met390Arg	39	M		X	X
GST07	*BBS1*	c.1169T>G/p.Met390Arg/ c.1169T>G/p.Met390Arg	35	F		X	
GST09	*BBS2*	c.1237C>T/p.Arg413*/ c.1237C>T/p.Arg413*	28	F	X		
GST20	*BBS10*	c.285A<T/p.Arg95Ser/ c.2119_2120del/p.Val707*	27	F		X	
GST25	*BBS10*	c.530A>G/ p.Tyr177Cys/ c.530A>G/ p.Tyr177Cys	19	M	X	X	X
GST27	*BBS1*	c.1169T>G/p.Met390Arg/ c.1169T>G/p.Met390Arg	60	F			X

GST28	*BBS10*	c.271dupT/p.Cys91Leufs*5/ c.271dupT/p.Cys91Leufs*5	43	F		X	X
GST36	*BBS1*	c.1708C>T/p.Arg570*/ c.1708C>T/p.Arg570*	34	F		X	
GST37	*BBS1*	c.1708C>T/p.Arg570*/ c.1708C>T/p.Arg570*	32	M		X	
GST38	*BBS1*	c.1708C>T/p.Arg570*/ c.1708C>T/p.Arg570*	24	M		X	
GOS1	*CEP290/ BBS14*	c.5167A>G/ p.Met1723Val/ c.-1003T>C/ 12:88142875	6	F	X		
GOSH2	*BBS2*	c.72C>G/p.Tyr24*/ c.823C>T/ p.Arg275*	16	M	X		

Table 7.3 Control human fibroblast primary cell cultures

Sample code	*Age*	*Gender*	*Used for ciliation and cilia length assays*	*Used for proliferation assay*	*Used for gene expression array*
CTRL1	F	40	X	X	X
CTRL2	F	32	X		X
7303	F	20		X	X
6823	F	25		X	
6824	M	38		X	
L1084	M	39		X	
L665	F	32		X	
6076	M	37		X	

Table 7.4 Urine derived Renal Epithelial Cells from BBS patients

ID							
GOSH3	Age	Gender	*Geno-Type*	*Mutation type*	*Renal phenotype*	*Used for ciliation and cilia length assays*	*Used for gene expression array*
GOSH4	11	F	*BBS10*	c.271dupT/p.Cys91Leufs*5/ c.271dupT/p.Cys91Leufs*5	Echogenic lobulated kidneys with no corticomedullary differentiation. CKD3	X	X
GOSH5	7	F	*BBS1*	c.48-1G>A/splicing at the intron 1/exon2 boundary/ c.1169T>G/p.Met390Arg	Hydronephrosis. CKD1	X	X
GOSH8	7	M	*BBS10*	c.271dupT/p.Cys91Leufs*5/ c.669_674dup/p.Gly224_Val225 dup	Nephrocalcinosis CKD1	X	X
ICH2	9	M	*BBS2*	c.175C>T/p.Gln59*/ c.416G>A/p.Gly139Asp	Enlarged kidneys. CKD1	X	X
GOSH13	8	F	*BBS1*	c.1169T>G/p.Met390Arg/ c.98dupA/p.Asn33Lysfs*66	Nil	X	X
GUYS3	36	M	*BBS7*	c.712_715del/p.Arg238Glufs*59 / c.712_715del/p.Arg238Glufs*59	Cystic dysplastic kidneys CKD2		X
GUYS4	39	M	*BBS10*	c.2119_2120del/p.Val707* / c.964_966del/p.Tyr322del	Nephrogenic diabetes insipidus. CKD5	X	X
GUYS8	32	M	*BBS1*	c.1169T>G/p.Met390Arg / c.1169T>G/p.Met390Arg	Nil	X	X

*CKD= Chronic kidney disease.

Table 7.5 Control Urine derived Renal Epithelial Cells

Sample code	*Age*	*Gender*	*Used for ciliation and cilia length assays*	*Used for gene expression array*
CTRL3	F	24		X
CTRL4	F	32		X
CTRL5	F	2		X
CTRL6	F	5		X
CTRL7	F	5	X	
CTRL8	M	30		X
CTRL9	F	20	X	
CTRL10	M	25		X
CTRL11	F	24		X
CTRL12	M	31		X

7.2.2 Cilia length and ciliation

7.2.2.1 Fibroblasts

7.2.2.1.1 Ciliation percentage correlates positively with confluence but does not discriminate between patient and control cells.

Ciliation correlates positively with the confluence index (mean number of nuclei per image multiplied by magnitude) in all cell cultures(r=0.77) (figure 7.2.a). A Mann-Witney U test did not find a significant difference in ciliation percentage between control and patient samples (p= 0.33), thus rejecting the hypothesis posed by previous researchers that cells derived from BBS samples are less likely to ciliate than those derived from control samples. However, on examination of individual cell cultures at different passages, increased confluence did not always correlate with increased ciliation rate although differences were small (figure 7.2.b)

7.2.2.1.2 Cilia length varies significantly in all samples and no consistent cilia length phenotype is identified in patient samples.

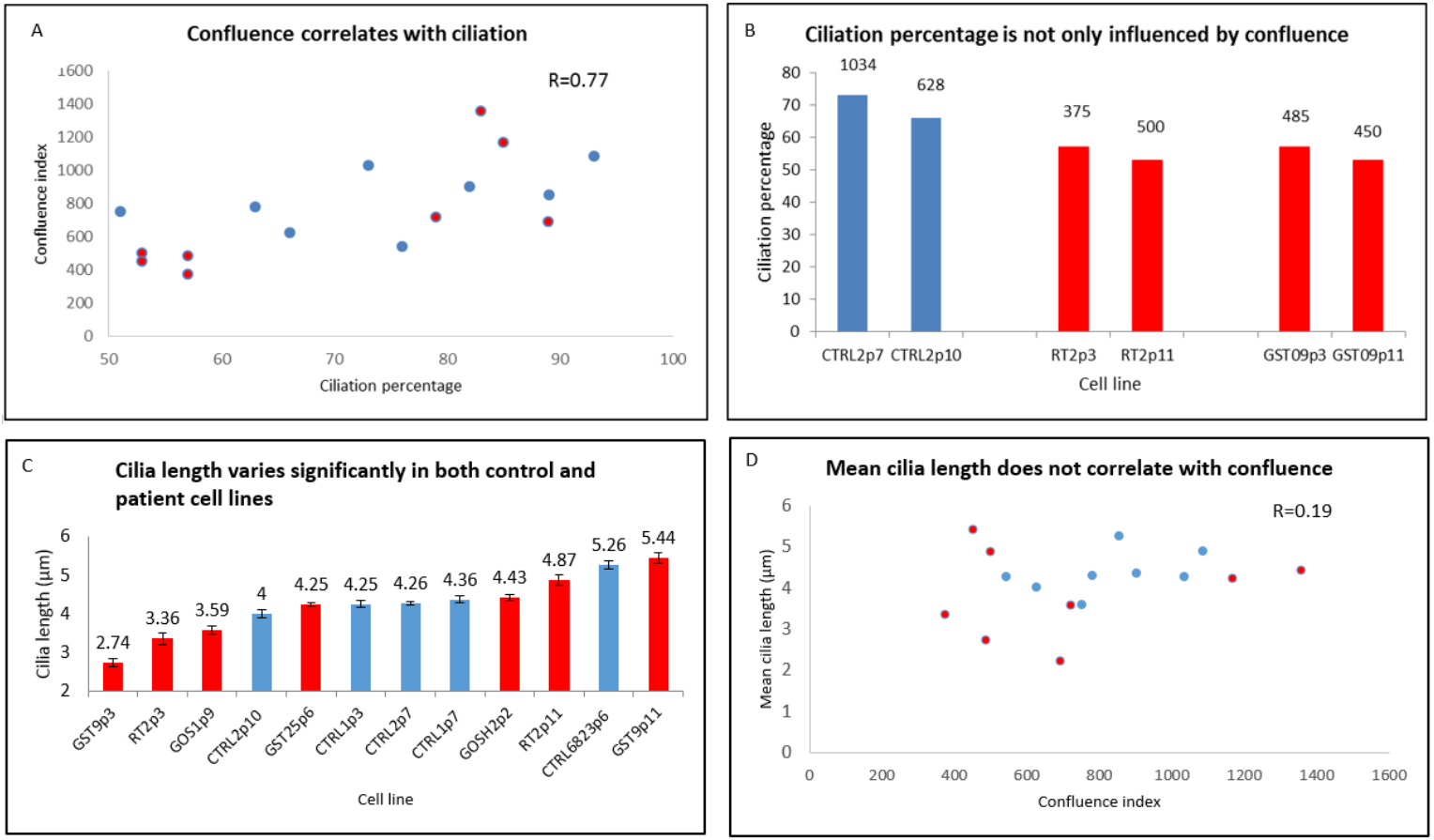
Mean cilia length varied significantly across all samples. Figure 7.3 shows representative immunocytochemistry images of all cell cultures assessed for ciliation and cilia length. In samples from BBS patients the mean length varied from 2.74µM to 5.44µM (mean: 4.09 SD: 0.85) and in control samples the cilia length varied from 4.00 to 5.26 (mean: 4.42, SD: 0.43) (figure 7.2.c). The variation in cilia length is greater in BBS samples. A Mann-Whitney U test did not reveal a statistically significant difference between the two groups (p=0.62). Cilia length did not correlate with confluence (r=0.19) (figure 7.2.d). Cilia length also varied within cell cultures cultured at different passages and did not consistently correlate with confluence (figure 7.2.e). There was no correlation between cilia length and ciliation (r=0.16) (figure 7.2.f).

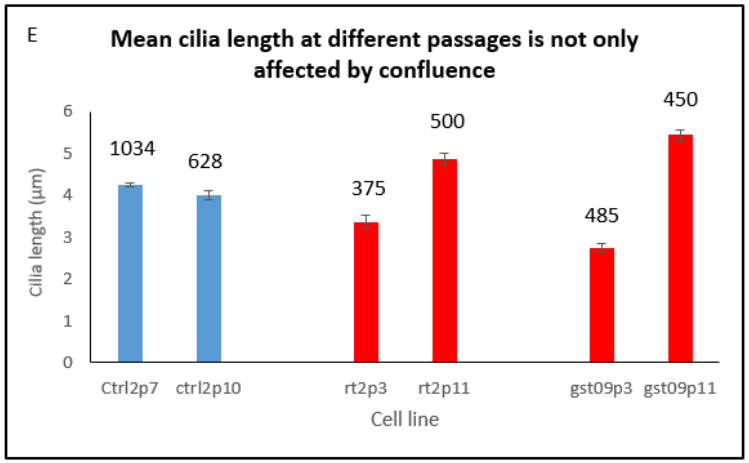
7.2.2.1.3 Ciliation percentage is smaller and mean cilia length is shorter in cells from a BBS14 (CEP290) patient compared to control and the phenotype does not resolve on treatment with purmorphamine.

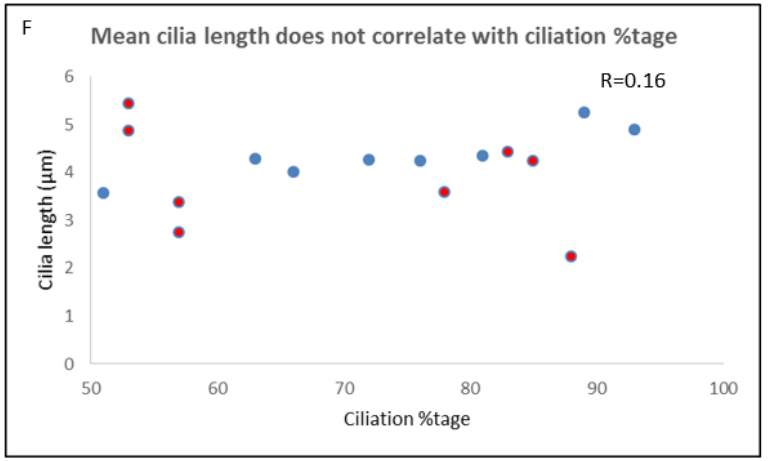
Fibroblasts from an eight year old patient with two novel mutations in *BBS14* (*CEP290*) demonstrated a clear reduction in mean cilia length (3.59µM versus 5.23 µM; P<0.0001) and ciliation in comparison to control samples (88% versus 78%). Incubation with 20µM Purmorphamine maintained a statistically significant difference in cilia length between the control sample and patient sample (4.89µM versus 2.23µM, P<0.0001) and ciliation level (93% versus 88%). Of note incubation with Purmorphamine decreased cilia length in both the control and patient samples (figure 7.2.g).

Figure 7.2 Ciliation and cilia length in fibroblasts.

BBS patient samples in red, control samples in blue. All error bars represent standard error. A. Increased confluence correlates with increased ciliation percentage (R=0.77). B: Ciliation fraction is not only affected by confluence. Numbers above each column indicate the confluence index (mean number of cells per image multiplied by the magnification). In the RT2 cell culture ciliation decreased despite the higher confluence in the passage 11 sample. C: Mean cilia length in ascending length. Considerable variability is evident in both control and patient samples. D: Mean cilia length does not correlate with ciliation (R=0.16).




E
Mean cilia length at different passages is not only affected by confluence
Cilia length (µm)
0
1
2
3
4
5
6
1034
628
375
500
485
450
Ctrl2p7
ctrl2p10
rt2p3
rt2p11
gst09p3
gst09p11
Cell line


F
Mean cilia length does not correlate with ciliation %tage
R=0.16
Cilia length (µm)
0
1
2
3
4
5
6
50
60
70
80
90
100
Ciliation %tage

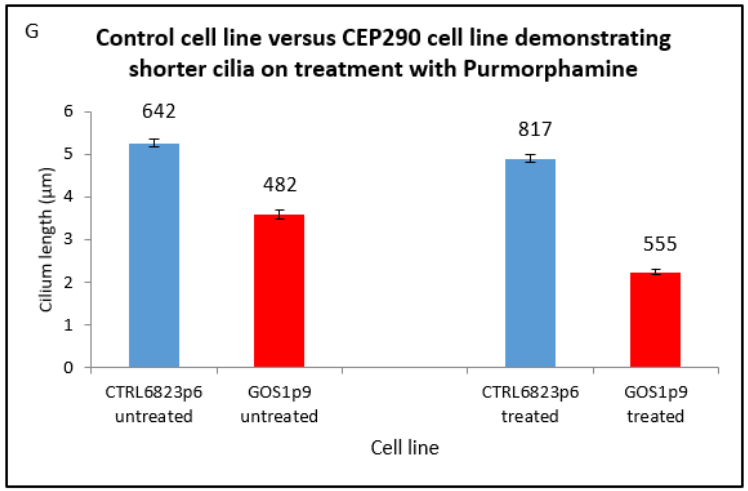
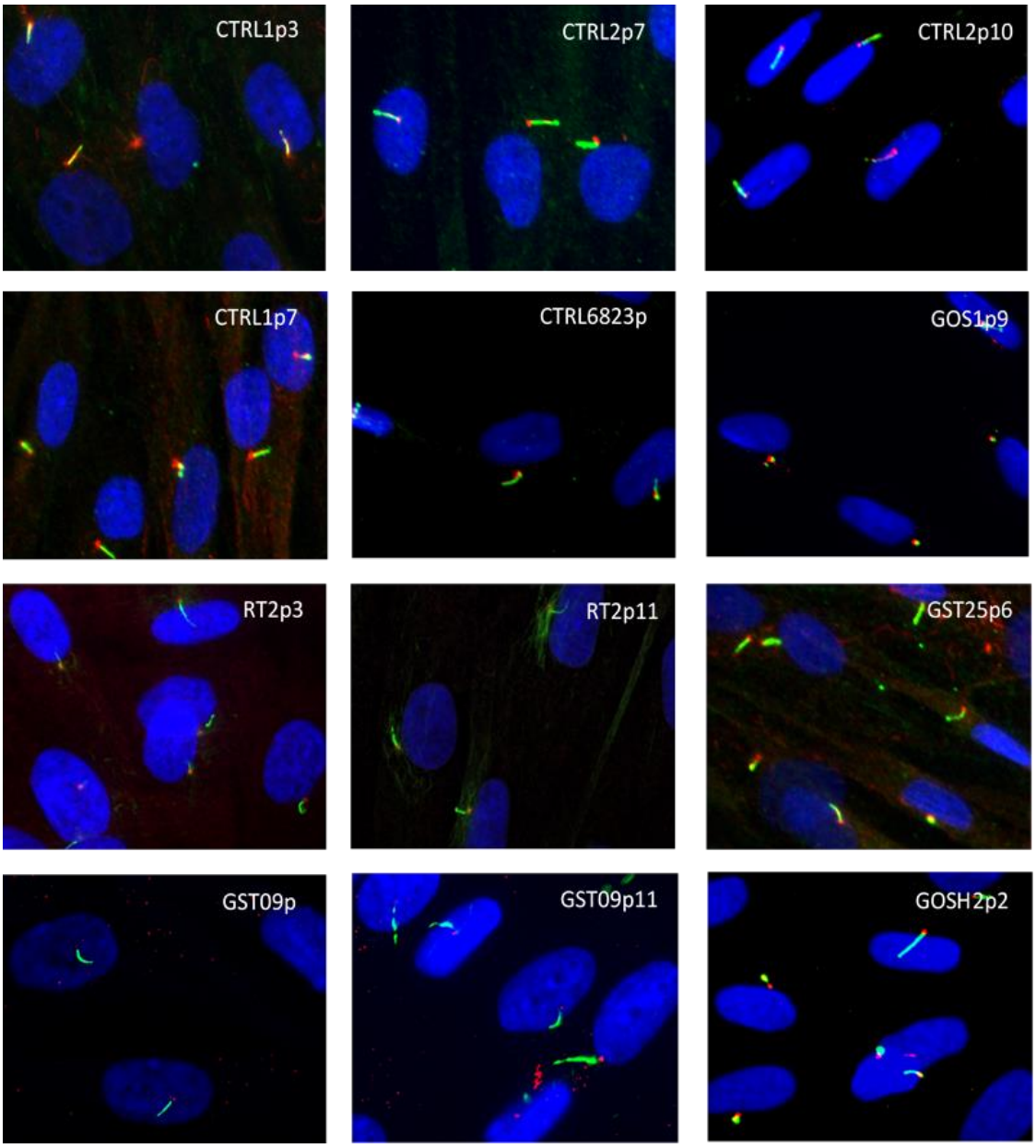

G
Control cell line versus CEP290 cell line demonstrating shorter cilia on treatment with Purmorphamine
Cilium length (µm)
0
1
2
3
4
5
6
642
482
817
555
CTRL6823p6 untreated
GOS1p9 untreated
CTRL6823p6 treated
GOS1p9 treated
Cell line

Figure 7.3 Representative immunocytochemistry images of all fibroblast cell cultures assessed for ciliation percentage and cilia length

Nuclei DAPI stained, Arl13B stained green and acetylated and gamma tubulin stained red. Except for GOS1, cilia length did not vary significantly between BBS and control fibroblasts.



7.2.2.2 Urine derived epithelial cells

7.2.2.2.1 Ciliation percentage is higher in BBS patient samples but varies significantly between samples.

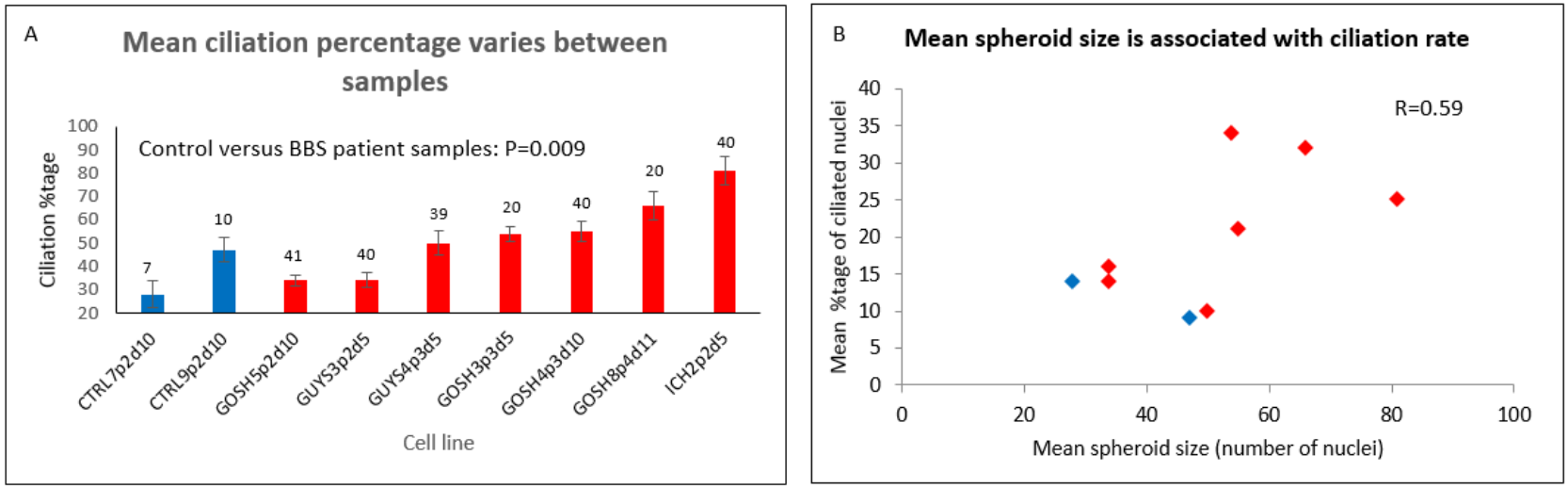
There was considerable difference in ciliation rate between and within samples. The control samples' ciliation rate varied between 28% and 47% and in patient samples the variation extended between 34% and 81% (figure 7.4.a). On a Mann-Whitney U test, there was a statistically significant difference in ciliation percentage between controls and patient samples (p=0.009). On calculation of the correlation coefficient between ciliation rate of each spheroid and cilia length the R value was 0.59 indicating a positive correlation. (figure 7.4.b).

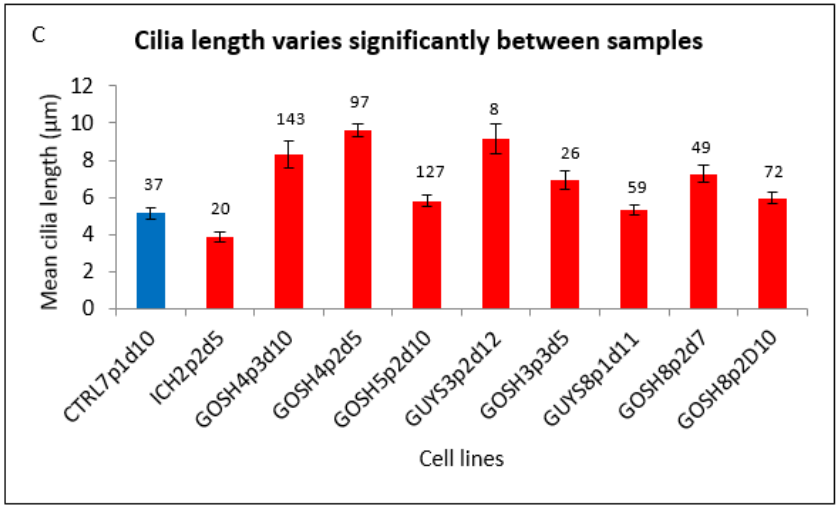
7.2.2.2.2 Mean cilia length varies between and within cell samples

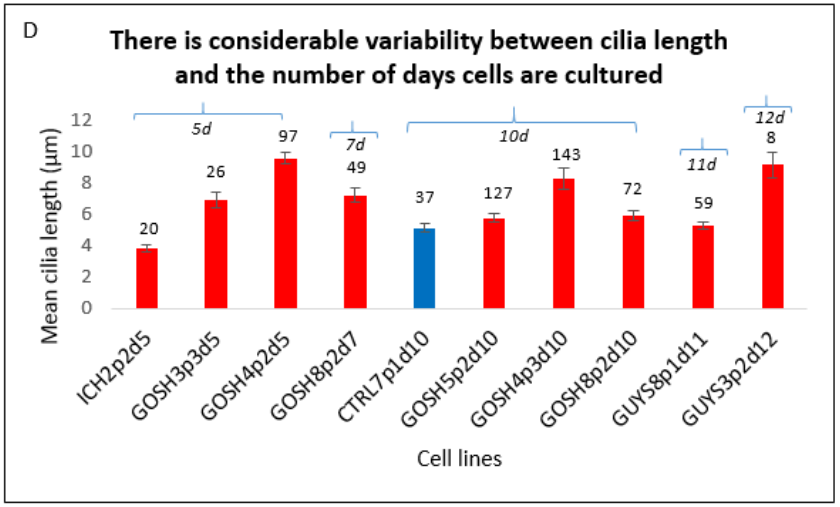
Figure 7.5 shows representative immunocytochemistry images of all UREC cell cultures assessed for ciliation and cilia length. Mean cilia length varied significantly across different samples (figure 7.4.c). In samples from BBS patients the mean cilia length ranged from 3.58 to 9.58µm. The control sample mean cilia length was 5.14µm. On comparing cilia length from patient samples versus the control sample the Mann-Whitney U test P value was 0.005. There was no consistent pattern on comparing samples according to the passage number or days cultured (figure 7.4.d). However, on evaluating two patient samples GOSH4 and GOSH8 cultured for different lengths of time (5 and 10 days and 7 and 10 days respectively) it is notable that cilia in both samples were shorter at day 10 than at day 5/7 (figure 7.4.e).

Figure 7.4 Ciliation and cilia length in urine derived renal epithelial cells

BBS patient samples are in red, control samples are in blue. All error bars represent standard error. A: Mean ciliation percentage varies significantly between samples. Control samples are statistically significantly less likely to be ciliated than patient samples. Number of spheroids assessed indicated by numbers on top of each column. B: Correlation between ciliation rate and spheroid size. C: Mean cilia length varies significantly between BBS patent samples. D: On comparing cilia length between samples cultured for different periods of time no clear pattern is evident. E: On comparing two samples grown for different lengths of time there is evidence of decreased cilia length at day 10 of culture compared to day 5/7.




C
Cilia length varies significantly between samples
Mean cilia length (µm)
0
2
4
6
8
10
12
37
20
143
97
127
8
26
59
49
72
CTRL7p1d10
ICH2p2d5
GOSH4p3d10
GOSH4p2d5
GOSH5p2d10
GUYS3p2d12
GOSH3p3d5
GUYS8p1d11
GOSH8p2d7
GOSH8p2D10
Cell lines


D
There is considerable variability between cilia length and the number of days cells are cultured
Mean cilia length (µm)
0
2
4
6
8
10
12
5d
7d
10d
11d
12d
20
26
97
49
37
127
143
72
59
8
ICH2p2d5
GOSH3p3d5
GOSH4p2d5
GOSH8p2d7
CTRL7p1d10
GOSH5p2d10
GOSH4p3d10
GOSH8p2d10
GUYS8p1d11
GUYS3p2d12
Cell lines

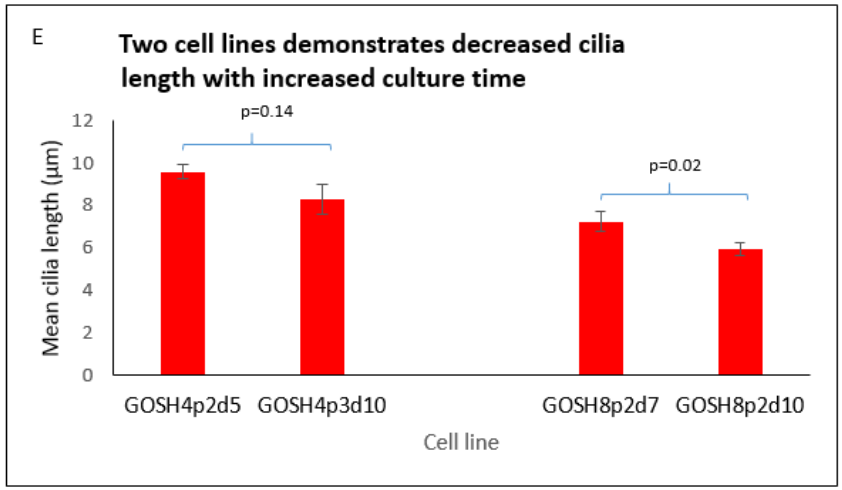
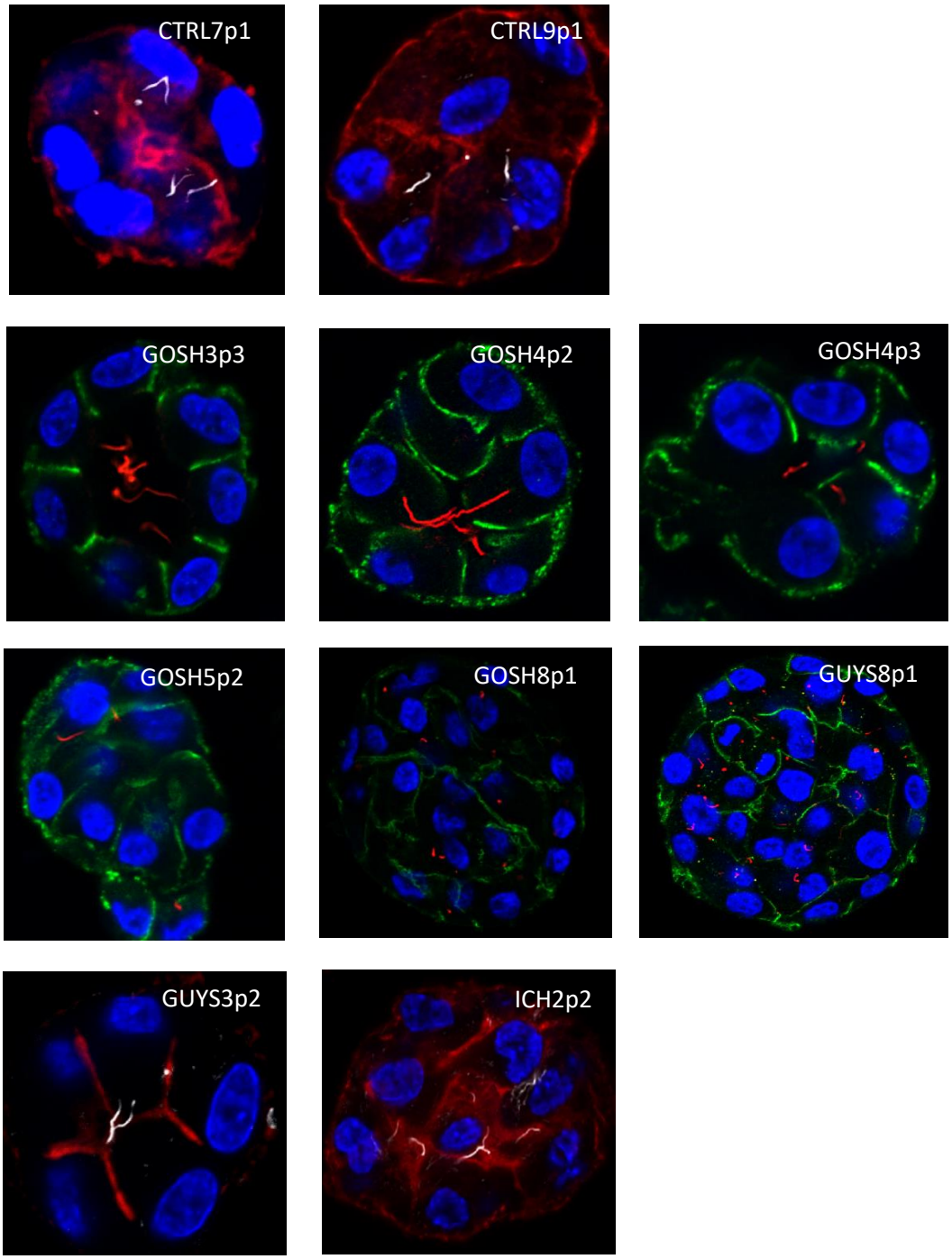

E
Two cell lines demonstrates decreased cilia length with increased culture time
Mean cilia length (µm)
0
2
4
6
8
10
12
p=0.14
p=0.02
GOSH4p2d5
GOSH4p3d10
GOSH8p2d7
GOSH8p2d10
Cell line

Figure 7.5 Representative immunocytochemistry images of all UREC cell cultures assessed for ciliation, cilia length and number of nuclei per spheroid

CTRL7, CTRL9, GUYS3, ICH2: nuclei DAPI stained, acetylated tubulin stained white, and β catenin stained red. GOSH3, GOSH4, GOSH5, GOSH8, GUYS8: Nuclei DAPI stained, acetylated tubulin stained red and β catenin stained green. Spheroids grown from GOSH8 and GUYS8 cell cultures grew considerably bigger than other samples.

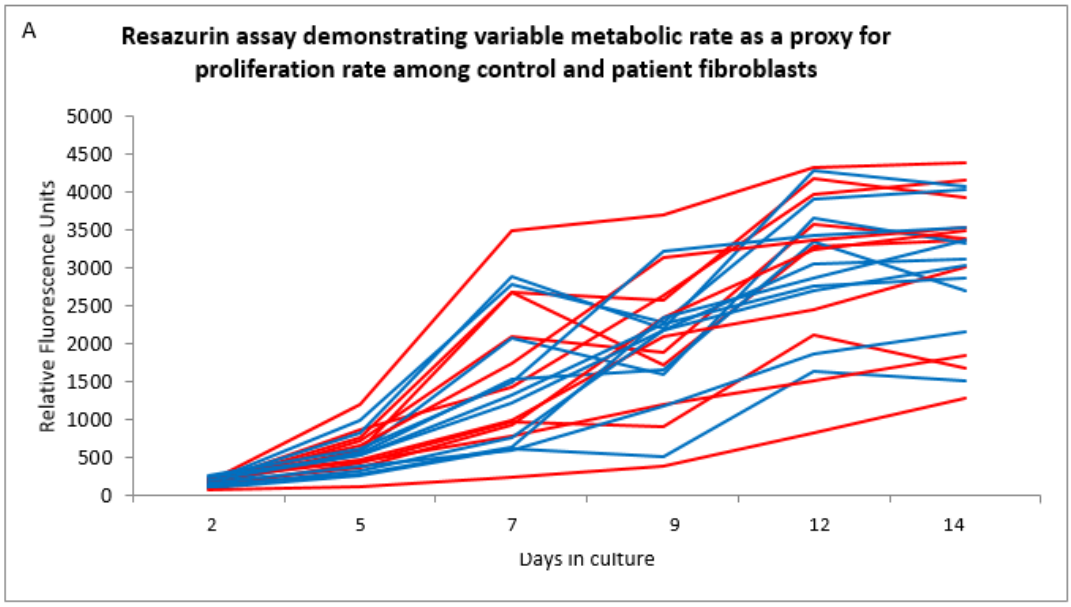


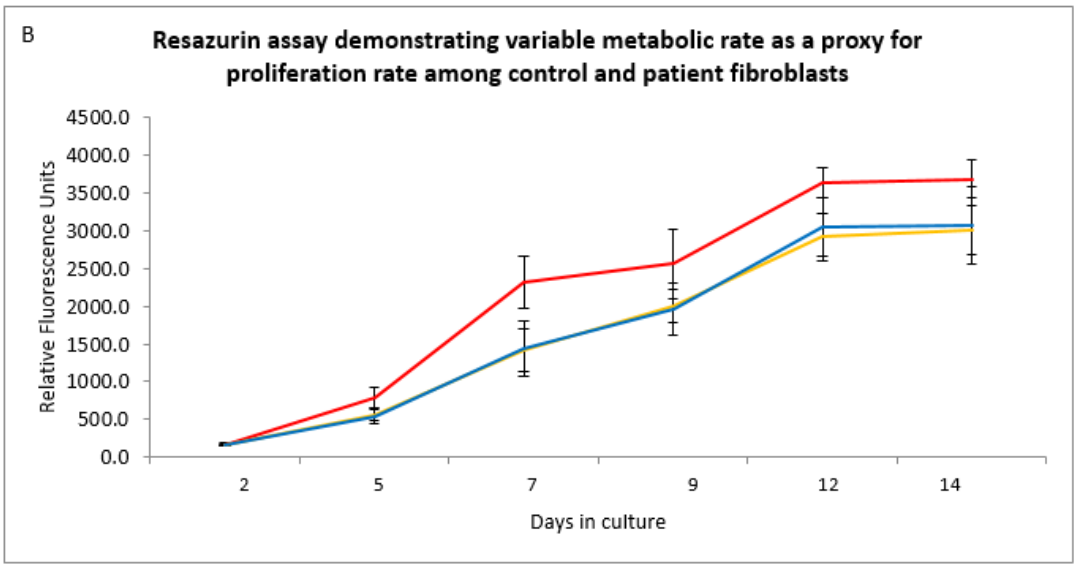
7.2.3 Cell proliferation

Resazurin measures cell metabolic activity as a proxy for cell proliferation (figure 7.6). Variability in cell proliferation rate in both controls and BBS patient fibroblast samples was evident. All cell cultures were cultured at passages 4-9 to ensure equitable proliferation conditions. However, on assessing the mean proliferation rate between three different groups (controls, *BBS1* and *BBS10* samples) it is clear that cells from patients with *BBS10* mutations proliferate at a higher rate than controls or samples from patients with *BBS1* mutations. However, a Mann-Whitney U test comparing mean Relative Fluorescence Units between controls, *BBS1* and *BBS10* samples did not reveal a statistically significant difference at any time point.

Figure 7.6 Resazurin assay as a proxy for fibroblast proliferation expressed in Relative Fluorescence Units (RFU)

A: Variable cell proliferation rate is evident in control(blue) and BBS patient samples (red). B: The mean proliferation rate according to genotype: control (blue), *BBS1* (yellow), *BBS10* (red). Error bars represent standard error.





7.2.4 Gene expression profiling

Gene expression aberrations in pathways directly implicated in the structure and function of primary cilia were examined using the Primary Cilia pathway RT2 profiler PCR array (PAHS-127Z; Sabioscience) assessing the expression of 84 genes (gene list and full details outlined in chapter 2). Fibroblasts and URECs from patients with BBS were

compared to controls. Fold regulation was considered significant if there was a fold change of 2 or more and a P value less than 0.05.

7.2.4.1 Fibroblasts

Table 7.6 demonstrates fold regulation on comparison of three control primary cell cultures of fibroblasts and six BBS patient samples (three *BBS1* and three *BBS10* samples). Four genes were found to be upregulated in BBS fibroblasts compared to controls and one gene was downregulated.

Fold regulated genes were primarily involved in different pathways except *IGF-1* and *FOS*. *IGF-1* is structurally and functionally similar to insulin but promotes greater growth activity. Expression of *IGF-1* has been shown to induce expression of transcription factor c-FOS [277,278]. However, in this case *FOS* has been downregulated.

Table 7.6 Primary cilia gene expression fold regulation in fibroblasts from BBS patients versus controls

Gene	Description	Role	**Fold regulation**
MKS1	Meckel syndrome, type 1	Centrosome migration during early ciliogenesis	3.33
VANGL2	Vang-like 2	Plays a role in early morphogenesis and is involved in planar cell polarity	2.71
MOS	V-mos Moloney murine sarcoma viral oncogene homolog	Serine/threonine kinase that activates the MAP kinase cascade through phosphorylation of MAP kinase activator MEK	2.27
IGF1	Insulin-like growth factor 1	Growth factor	2.17
FOS	FBJ murine osteosarcoma viral oncogene homolog	Forms complex with JUN/AP1 transcription factor	-2.17

7.2.4.2 URECs

Table 7.7 demonstrates the down regulated genes in URECs, FOS, a transcription factor implicated in cell proliferation, differentiation and apoptosis, was downregulated in patient samples with a fold change of -11, but no other cell cycle proteins were dysregulated.

Upon mapping the other downregulated genes, several genes were involved in the Wnt/ planar cell polarity pathway (*PKD1, BTRC, FAT4, VANGL2*), whereas *SMO* and *GLIS2* are involved in the hedgehog signalling pathways.

There was no evidence for the implication of any single or combination of ciliary specific pathways on comparison of gene expression in BBS samples versus controls.

Table 7.7 Primary cilia gene expression fold regulation in URECs from BBS patients versus controls

Gene	Description	Role	Fold regulation
BTRC	Beta-transducin repeat containing E3 ubiquitin protein ligase	Mediates ubiquitination of a number of molecules and participates in Wnt signalling	-2.07
LRP2	Low density lipoprotein receptor-related protein 2;	Mediates HDL endocytosis	-2.12
ADCY3	Adenylyl cyclase 3	Catalyzes the formation of the signaling molecule cAMP in response to G-protein signalling	-2.12
ARL13B	ADP-ribosylation factor-like 13B	Maintains the association with IFTA and IFTB subcomplexes in intraflagellar transport.	-2.16
BBS7	Bardet-Biedl syndrome 7	Forms part of the BBSome bringing membrane proteins to the cilium.	-2.17
FAT4	FAT tumour suppressor homologue 4	Plays a role in maintenance of planar cell polarity	-2.29
GLIS2	GLIS family zinc finger 2	Hedgehog signalling pathway repressor.	-2.34
ADCY7	Adenylate cyclase 7	Catalyses the formation of signalling molecular cAMP in response to G protein signalling.	-2.38
PKD1	Polycystic kidney disease 1	Involved in renal tubulogenesis, acts as a regulator of cilium length	-2.66
SMO	Smoothened, frizzled family receptor	G protein coupled receptor transducing hedgehog signalling.	-2.67
VANGL2	Vang-like 2	Plays a role in early morphogenesis and is involved in planar cell polarity	-2.71
FOS	FBJ murine osteosarcoma viral oncogene homologue	Forms complex with JUN/AP1 transcription factor	-11.63

7.3 Discussion

Fibroblast primary cell cultures were derived from patients aged 6-60 years with a male: female split of 8:9. The genotype distribution of samples used for the purposes of these experiments (n=17) roughly reflected the genotype prevalence in the population: eight *BBS1* samples, four *BBS10* samples and four *BBS2* samples, and one less common

genotype: *CEP290* (*BBS14*). All patient samples, except the CEP290 sample, were derived from adults. Control fibroblast samples were all derived from adults ranging in age from 20 to 40 with a slight preponderance of samples from females (male to female ratio 5:3).

Only around 30% of harvested UREC samples proliferate to form a primary cell culture[73]. The UREC primary cell cultures that proliferated and were used for the purposes of this experiment were derived from patients aged seven to 39 with the majority of samples derived from children (n=6). Six samples were derived from males and three from females. The genotype distribution of samples that proliferated and could be used for this study (n=9) did not reflect the prevalence in the BBS population: There were three *BBS1* samples, four *BBS10* samples, one *BBS2* sample and one *BBS7* sample. There was also a higher prevalence of structural renal abnormalities than expected based on the findings reported in chapter 3 and reported by Forsythe *et al*[1]. The higher prevalence of non-*BBS1* genotypes and renal structural aberrations amongst the UREC samples that proliferated is an interesting finding as it may represent a similar aberrant cell cycle reflected in the proliferation observed in the Resazurin assay on patient fibroblasts where samples from patients with *BBS10* mutations proliferated at a higher rate than controls or *BBS1* samples. It may also reflect the poorly controlled proliferation that has been shown to lead to renal disease and cystogenesis in ADPKD[128,259].

Cilia length and ciliogenesis: The majority of published work assessing the cellular phenotype in BBS is based on cell cultures derived from knockout mouse models. This may skew the cellular phenotype which may be more severe than the phenotype observed in humans where variable levels of protein production may be preserved. Cell cultures from animal models are also often derived from tissues relevant to the

phenotype (primarily renal tissue), which may affect the presence or absence of a cilia phenotype. Of note, there is no reported dermal phenotype in BBS, although it has been suggested that an increased number of dermal naevi may be present.[279].

In fibroblasts confluence is known to be the major initiator for ciliation as it facilitates entry into G0 of the cell cycle. This study supports the notion that confluence is a major cellular incentive for ciliation as both control and patient cell cultures appear to follow the same positive correlation between confluence and ciliation. However, other factors contribute since ciliation percentage varies between and within cell cultures out of sync with the degree of confluence. Additional factors are likely to include passage and environmental factors such as minor variations in serum starvation time and composition of serum starvation medium.

The strikingly short cilia and poor ciliation in the *CEP290* (*BBS14*) sample corroborates evidence that *CEP290* mutations in conjunction with a renal phenotype is associated with a structural ciliary phenotype. However, the findings here are contrary to those reported by Srivastava[74] and Shimada[228]. Although they also observed lower ciliation percentage they report that patient samples from individuals with *CEP290* mutations in conjunction with a renal phenotype had longer cilia than control samples. Furthermore, they found that the cilia length phenotype was corrected on treatment with Purmorphamine. The cilia length deficit demonstrated by the *CEP290* sample used in this study did not resolve on treatment with Purmorphamine. On the contrary, cilia were even shorter in both the patient and control sample following Purmorphamine treatment indicating that Purmorphamine may have a detrimental effect on cilia length in general rather than specifically rescuing the *CEP290* cilia phenotype.

In URECs the statistically significant difference in ciliation and cilia length between the control sample and patient samples point to a possible cellular phenotype in URECs. The phenotype may represent a true difference between controls and patient samples or it may be a function of the poorer proliferation of control samples compared to patient samples making them less likely to ciliate and grow long cilia. The variability between samples is high which makes it a less attractive phenotypic readout for therapeutic intervention. There was no obvious relationship between the presence of renal pathology and the UREC cellular phenotype. It is notable that the two patient cell cultures GOSH4 and GOSH8 cultured for different lengths of time (5-10 days and 7-10 days respectively) showed significantly longer cilia at 5/7 days culture than the control sample and shorter at 10 days culture in length. This is in contrast to the results demonstrated by Mokrzan et al[227] where cilia in renal cell samples from *bbs4* knockout mice demonstrate shorter cilia at 5 days and longer cilia at 10 days as compared to controls. This may reflect differences between mice and humans as well as differences in the cell type and genotype variations.

URECs offer an attractive model for BBS as they are both easily accessible through urine collection and offer a relevant cellular model given the frequency of renal disease in BBS. However, the lack of a consistent phenotype makes this an inconvenient candidate biomarker for disease and specifically for measuring therapeutic intervention.

Proliferation: This study demonstrates considerable variability between patient cell cultures and control cell cultures. Although not statistically significant, *BBS10* cell cultures appear, on average, to be hyperproliferative in comparison to control samples, whereas *BBS1* cell cultures on average proliferate at a similar rate to control samples.

This is in keeping with the hypomorphic clinical phenotype. However, the variability between both samples and controls limits the use of this phenotype.

Previous studies have suggested that proliferation may be greater in BBS cells than controls. Hernandez-Hernandez et al[225] demonstrated that cells derived from knockout *Bbs4* and *BBs6* mice had a higher proliferation rate than controls on scratch- wound assay[225]. Furthermore, Aksanov *et al* demonstrated that *BBS4* silenced pre-adipocytes proliferate at a significantly higher rate than control samples and that on re-introduction of *BBS4* the proliferation rate returns to that of control cells[267].

Although the role of several cilia proteins in the cell cycle is has come to light, the contribution of BBS proteins in the cell cycle remains largely unexplored.

Gene expression: Gene expression differences in BBS versus patient fibroblasts demonstrated upregulation of *MKS1, MOS, IGF1, VANGL2* and down regulation of *FOS*. Whilst *MKS1 (BBS13)* is involved in centriole migration and ciliogenesis, *MOS* is a serine/ threonine kinase that activates the MAP kinase cascade[280]. *IGF-1* promotes many of the growth mediating effects of growth hormone and is associated with cellular hyperproliferation[281]. *VANGL2* is a planar cell polarity protein that promotes cell directionality[282]. Upregulation of *MOS* and *IGF-1* may reflect the average increased proliferation observed in BBS fibroblasts.

On examining gene expression in URECs the following genes were downregulation: *BTRC, LRP2, ADCY3, ARL13B, BBS7, FAT4, GLIS2, ADCY7, PKD1, SMO, VANGL2.* This includes members of the Wnt/PCP pathway and hedgehog signalling pathways, both of which have been associated with the development of polycystic kidney disease.

Downregulated genes participating in the Wnt/ PCP signalling pathway are implicated as follows: Wnts bind to the extracellular domain of *PKD1* and induces whole cell current and Ca2+ influx[127]. *VANGL2* is required for normal structural development of the kidneys[283]. *BTRC* is associated with β-catenin destruction motifs[284]. Interestingly, mutant *FAT4* mouse models display a polycystic kidney phenotype and this is accentuated if the mice are additionally haploinsufficient for *VANGL2*[285]. Consistent with the downregulation of planar cell polarity proteins a previous study has also demonstrated evidence of polarity aberrations in BBS cells[225].

SMO and *GLIS2* are members of the hedgehog signalling pathway. Hedgehog signalling has been found to be upregulated on acute kidney injury[286] or where there is evidence of renal fibrosis[269,287] and downregulation of this pathway has previously been shown to be protective against further kidney injury[288].

It is likely that multiple signalling pathways are involved in predisposing BBS kidneys to structural and functional aberrations[289]. Therefore, deciphering the causative signalling pathway disruption from cells extracted at various stages of renal pathogenesis may yield limited answers.

Of note, *LRP2* is downregulated in patient samples in comparison to controls. This is specifically of interest to the clinical phenotype as LRP2 mediates HDL endocytosis and patients with BBS tend to have low HDL cholesterol and high LDL cholesterol [own unpublished data].

ADCY3 was also downregulated. Wong et al[290] demonstrated that *ADCY3* mutant mice had poor olfaction and Grarup et al[291,292] identified that splice site variants in *ADCY3* in the Greenlandic population are associated with increased BMI and waist circumference.

Both of these associations may contribute to the pleiotropic effects observed in patients with Bardet-Biedl syndrome.

FOS is downregulated in both the patient fibroblasts and URECs. This is a surprising finding in this context since BBS cells on average are more proliferative than control cells. Rankin et al[293] demonstrated that cultured cells from mouse model (C57BL/6J-cpk) cystic kidneys overexpress *c-FOS* consistent with hyperproliferation. Of note, it has been shown that *c-FOS* expression is very responsive to variable levels of FBS in culture medium[293] which may provide an explanation. It is more likely, however, that the downregulation represents a real change given that is evidenced in both types of cells and is substantially downregulated in both cell types, in particular the urine derived renal epithelial cells.

Downregulation of BBSome component *BBS7* and IFT associated protein *ARL13B* could reflect downstream adverse effects of poorly functioning/ lacking protein components of the BBSome/ chaperonin complex in patient URECs. Downregulation of *ADCY7* could reflect poorer cAMP signalling.

These findings corroborate the hypothesis that multiple signalling pathways are disrupted in the ciliopathy renal phenotype based on studies on ADPKD[129] but may also reflect the diversity of renal disease in the patients from whom the URECs were derived.

Limitations The biological variability between the control and patient samples used in this study is an advantage in that it provides a cellular representation that mirrors the phenotypic variability observed in the clinical setting. However, it is also a limitation as a large sample group is required to demonstrate a phenotype and the risk of type 1 and type 2 statistical errors is inherently higher. Due to logistical arrangements in retrieving patient samples and limitations of URECs culture methods it was only possible to acquire

paired fibroblast and UREC samples from one individual. Having a matched cohort could have limited analysis aberrations relating to patient specific cilia length and ciliation as well as gene expression.

Due to the diversity of genotypes and variability in renal pathology it was not possible to conduct genotype-phenotype analysis in the fibroblasts and URECs or assess for aberrations in gene expression in samples from patients with renal disease versus no renal disease.

A further limitation is the lack of available biologically important tissue for assessment. Rod-cone dystrophy is a hallmark of Bardet-Biedl syndrome, however, establishing primary retinal cell cultures from patients is restricted by practical and ethical issues.

The limited number of available cultured control UREC samples, particularly for the purposes of examination of cilia length and ciliation, is a clear limitation in this study, as control sample URECs were less likely to proliferate.

Although a two-fold change in gene expression and P value of <0.05 are scientifically accepted cut-off points this may not accurately translate into biologically meaningful changes. This may highlight gene expression differences which are not biologically meaningful and miss out expression changes that are biologically significant but do not reach the two fold expression change threshold.

A limitation of studying cilia structure and function in cultured cell cultures is the unknown effect of exposing cells to effects of the culture medium. The potential for misleading results is highlighted by the finding that *FOS* expression is influenced by FBS concentration in the culture medium.

Further work is required to understand the cilia phenotype in BBS. The significant variability between patient samples hampers the identification of a clear phenotype which can be used for risk stratification and assessment of therapeutics.

Further work could include assessing localisation of G protein coupled receptors which are known to depend on the BBSome including Somatostatin Receptor 3 (*SSTR3*), Melanin-Concentrating Hormone Receptor 1 (*MCHR1*) and Dopamine Receptor 1 (*DR1*)[11] in patient versus control cilia in URECs and fibroblasts.

The cell culture process for URECs should be optimised. Currently the expected success rate of establishing a primary cell culture is 30%[73] in contrast to the success rate attained in establishing fibroblasts cell cultures which was 100% in this study.

Further work is required to clarify the role of BBS proteins in the cell cycle. This could be addressed through flow cytometry comparing the number of cells in different parts of the cell cycle in controls versus BBS patient cells. Given the *BBS1* hypomorphic clinical phenotype as well as the hypomorphic cell proliferation phenotype identified in this study, it would be of interest to explore genotype- phenotype correlations in BBS patient samples further and correlate these with the clinical phenotypes.

To fully ascertain differences in gene expression a greater number of samples should be collected with a potential for whole genome sequencing or SNP array sequencing. This would allow for more detailed insight into aberrations in gene expression. The primary cilia signalling pathways cannot be considered in isolation but interact with a multitude of other cellular pathways.

Acquiring a bigger sample size with more diverse phenotypes would allow for more detailed genotype-phenotype correlations.

Further work should be done to establish a relevant tissue specific cellular phenotype. One such avenue already in process includes differentiating induced pluripotent stem cells into optic discs. This would allow for detailed characterisation of a cellular phenotype in a relevant tissue and also for assessment of the effect of novel therapeutic agents or repurposed drugs.

This study demonstrates that the phenotype in cell cultures derived from animal models and commercial cell cultures does not always reflect the human cellular phenotype. Although it has identified possible candidate cellular phenotypes including cilia length and ciliation in CEP290 (*BBS14*) and cell proliferation in samples from BBS10 patients, neither of these phenotypes are consistently seen in samples from BBS patients. A novel molecular indicator for assessment of efficacy of therapy intervention should ideally be both consistently evident in samples from different patients and sufficiently contrasting the wild type phenotype in order for partial rescue of the phenotype to be quantifiable. Further work is in progress to build on these findings in order to identify a consistent cellular phenotype and molecular indicator in BBS patient cell cultures.

8 The effect of exercise on cognition in Bardet-Biedl syndrome

"What can I do now to help my child?

8.1 Introduction

Cognitive impairment is a major feature of the BBS phenotype and can be a source of significant debilitation. Nonetheless, this aspect of BBS has received limited attention, and until recently relatively little had been published describing the phenotype or natural history of cognition in BBS. Anecdotal evidence from the UK National BBS clinics suggests that many individuals experience learning and memory deficit as well other related social and communication skills difficulties. Unfortunately, other than psychological support, there is currently no intervention available to alleviate these difficulties.

8.1.1 Cognition and memory in BBS

In recent years there has been an increasing research interest in investigating the psychological and cognitive phenotype as well as brain morphology in patients with BBS[50,51,294,295]. On assessment of 109 patients, Beales *et al* reported that 50% had developmental delay and 62% were reported by parents to have learning difficulties[49]. Another study evaluating 21 children found that intelligence quotient (IQ) attainment was highly variable in this patient group, but most were in the spectrum of borderline to mild learning difficulties with IQs ranging between 42 and 108[50,51].

Kerr *et al*[296] investigated the cognitive and behavioural phenotype of 24 individuals with molecularly confirmed BBS ranging in age from six to 38 years of age and found that the mean intellectual functioning fell 1.5 SD below normal, although the majority of

individuals could not be classified as having intellectual disability. Memory performance was highly variable and statistically significantly lower than the norm with reference to auditory recall[296]. A study by Brinckman *et al*[51] assessing 42 patients with BBS in the age range 2-61 years found that when patients were assessed for memory function using the Rey Auditory Verbal Learning Test, the mean was within normal range but significant variability existed. A limiting factor when assessing memory in BBS in adults is the exclusive reliance on auditory tests due to poor visual function in the majority of patients. This limits the sensitivity and range of memory tests available for this population group.

Consistent across the studies that have examined the BBS cognitive phenotype is the prevalence of social and communication difficulties[50-52]. Brinckman *et al* found that particular areas of cognition deviating from the norm included symptoms of autism (77%), impaired functional independence (74%), attention deficit (69%) and severe impairment in perceptual reasoning (53%)[296]. Studies in BBS mouse models support the BBS neuropsychological phenotype demonstrating reduced social dominance and anxiety related responses [132,147].

8.1.2 The brain and BBS

Several studies have investigated the brain morphology of patients with BBS in order to clarify whether anatomical aberrations underlie the functional deficits in cognition. Davis *et al* studied the brains of *Bbs1*, *Bbs2*, *Bbs4* and *Bbs6* mouse models and found a number of neuroanatomical aberrations including reduced volume of the hippocampus and corpus striatum, thinning of the cerebral cortex and enlargement of the third and lateral ventricles[147].

Baker *et al* investigated 10 patients with BBS and compared them to age and gender matched healthy controls. MRI brain studies revealed reduced hippocampal volume and reduced grey matter volume. Two patients also had ventriculomegaly.

Bennouna-Greene *et al*[52] demonstrated that 11 of 26 patients with BBS had evidence of bilateral or unilateral hippocampal dysgenesis, but there was no correlation with neuropsychiatric phenotypes.

Another case-control study comparing 21 patients with BBS to healthy controls found reduced grey matter in subcortical regions only, and increased cerebrospinal fluid but was unable to comment on hippocampal volume[294].

Across these studies the most consistent feature is the hippocampal volume loss and dysgenesis. The primary function of the hippocampus is memory and learning[297]. Studies attest to the role of the hippocampus for recall memory specifically, whereby patients with relatively selective bilateral hippocampal pathology show impairment in recall with intact recognition memory[298,299].

8.1.3 Cilia, memory and the hippocampus

Several lines of investigation indicate that normal cilia function is required for learning and cognition but also for the formation of memory[300,301].

A number of G protein coupled receptors (GPCRs) known to be associated with psychiatric disease and cognition localise to neuronal cilia in particular regions of the brain, and localisation is disrupted in ciliopathy animal models. Agassandian *et al* demonstrated that in a *Bbs4-/-* mouse model Adenylyl cyclase III (AC3) staining is preferentially lost in the hippocampus and amygdala[301]. Serotonin Receptor 6 (Htr6),

Somatostatin receptor 3 (SSTR3) and Dopamine-1 (DA1) all localise to the cilium and the latter two are known to interact with the BBSome[11,301]. Disrupted-In-Schizophrenia-1 (DISC1) localises to the centrosome and interacts with BBS4[302].

The requirement for normal cilia for memory acquisition is supported by the finding that hippocampal volume deficit is a distinguishing brain morphological feature in BBS[52,295]. A study investigating gene expression of *BBS1-12* healthy adults demonstrated that these BBS proteins are expressed at higher levels in the hippocampus compared to total brain expression except for *BBS1* which is expressed at a relatively lower level in the hippocampus[52]. This could suggest that *BBS2-12* are specifically required in the hippocampus.

Furthermore, cilia have been implicated in adult neurogenesis in mice in the subgranular zone of the dentate gyrus[301]. It has been suggested that defective cilia lead to altered sonic hedgehog signalling which leads to hippocampal dysgenesis in BBS mouse models highlighting the hippocampus as a key structure affected in ciliopathies[52,301].

8.1.4 The effect of exercise on the hippocampus

There is compelling evidence supporting the effect of aerobic exercise on hippocampal neuroplasticity and improved functional outcomes[303-305]. The majority of this evidence is derived from elderly patients with or at risk of developing Alzheimer's disease and charts the neuroprotective effect of aerobic exercise on hippocampal volume[306]. Studies attesting to the pro-neuroplastic effects of exercise on the hippocampus in children and young adults support this theory[297,307].

The underlying mechanisms for neuroplasticity have been extensively investigated in animals and appearbal to correlate with increased cerebral blood flow, increased local

production of insulin-like growth factor-1 (IGF1), brain-derived neurotrophic factor (BDNF), and its associated receptor tyrosine kinase B (trkB)[305]. Evidence from rodents suggests that running increases the number of dendritic spines and dendritic complexity in the dentate gyrus[308]. Angiogenesis may also contribute. [304,309] Compelling evidence from mouse hippocampal neurons suggests that cilia assembly is intrinsically linked with neuronal maturation[310]. Unpublished evidence from the Cilia Disorders Laboratory group at The UCL Great Ormond Street Institute of Child Health demonstrates that BBS mouse models have poor dendritic spine density in comparison to healthy controls and that dendritic spine density increases dramatically when BBS mice are primed to exercise for two or more weeks [personal communication, Dr Sonia Christou-Savina]

8.1.5 Assessing hippocampal volume change

Traditionally hippocampal volume change has been assessed computationally by manually tracing regions of interest (ROIs) on MRI brain scans and subsequently calculating volume changes[311]. This technique is time consuming, subject to missing small changes and operator dependent. This study employs a different technique; Voxel Based Morphometry (VBM), whereby every MRI image is registered to a template, thus reducing the effect of differences in volume between participants[311]. Statistical mapping is performed at each voxel and brain images are compared for each voxel. Maguire *et al*[312] performed the first high profile study using VBM to investigate hippocampal volume, comparing differences in volume between London black cab drivers and controls. A major advantage of VBM analysis is the elimination of operator dependent bias as the process is fully automated. However, VBM is subject to a number of possible confounding variables including automated misalignment and misclassification of tissue types and/ or structure[311].

Supporting evidence for hippocampal volume changes can be ascertained computationally by assessing for changes in diffusion and perfusion to an area of interest. Diffusion Tensor Imaging (DTI) offers a variation on conventional Magnetic Resonance Imaging, assessing the tissue –water diffusion rate[313]. DTI can provide information on fractional anisotropy (FA), whereby directionality of diffusion can be inferred using Tract-Based Spatial Statistics (TBSS)[116] as an indicator of brain changes related to development, degeneration and disease[313] (further details can be found in chapter 2). Radial Diffusivity (RD) is more specific for white matter pathology and appears to be modulated by myelin[314]. Arterial Spin Labelling (ASL) measures tissue perfusion by magnetically labelling blood water protons as a freely diffusible intrinsic tracer[315]. The advantage of ASL over traditional methods for assessing perfusion is that this offers a non-invasive approach to quantitation of blood flow[315].

8.1.6 Assessing hippocampal function in BBS

The hippocampus has been the subject of extensive research particularly in relation to its mnemonic effects. The importance of the hippocampus in recall memory, where information is retrieved, is well established[316,317] but controversy exists over the possibility that the hippocampus may also play a role in recognition memory[318 316]. Patai *et al*[316] performed a cohort study on 29 individuals with variable degrees of hippocampal atrophy sustained due to hypoxic or ischaemic injury in the neonatal/ perinatal period demonstrating that the extent of hippocampal atrophy correlates with the degree of recall memory deficit but not recognition memory. This is in keeping with several other smaller scale studies[317,319,320]. This provides the basis for the 'Pair Games' assessment used in this study where the primary outcome measure is recall memory.

Woollett and Maguire[321] demonstrated that the hippocampus is also involved in spatial memory, in particular complex spatial navigation. Multiple studies attest to the finding that London black cab drivers, who have extensive spatial navigational memory training, have large posterior hippocampi but comparatively smaller anterior hippocampi[312,321]. Anterograde associative memory, whereby new information associated with visual cues is retained, is poorer in London taxi drivers than in controls[321]. It has been hypothesised that spatial memory localises to the posterior hippocampus and associative memory localises anteriorly[321].

8.1.7 Assessing fitness in children with BBS

Assessing fitness in children presents a challenge, and in this population it is compounded by learning difficulties. There is no precedent for assessing fitness in children with BBS. The 10 metre incremental shuttle walk test (10m-ISWT) is designed to mimic a cardiopulmonary exercise test, and requires the patient to walk/ shuttle/ run along a 10 metre track in a time frame set to auditory beeps which become incrementally more frequent[322]. The 10m-ISWT has been shown to be a valid and reliable test in other paediatric populations[322,323].

8.1.8 Rationale

Aerobic exercise has been shown to induce hippocampal neuroplasticity in the elderly with dementia and in healthy adults and children. This study is the first to investigate if a behavioural intervention in the form of weekly supervised physical exercise can induce hippocampal volume expansion in a population with genetically determined hippocampal volume deficits.

8.2 Results

Eleven patients were recruited to the exercise intervention and five patients to the control arm. Figure 8.1 demonstrates the recruitment process and available data for the intervention and control groups. Tables 8.1 and 8.2 outline baseline data of the participants in each group. One patient in the intervention group did not complete the MRI brain scan at assessment two and one patient acquired fixed braces during the intervention and the subsequent MRI brain images were rendered unsatisfactory. The control and intervention groups had similar age ranges, gender split, genotypes and mean number of months between assessments.

Table 8.1 Intervention group demographics

Participant number	***gender***	***Age***	***Genotype***	***Time interval in months between assessments***
01*	F	11	*BBS1*	8.8
02	F	8	*BBS10*	6.7
03	F	9	Clinical	9.2
04	F	7	*BBS10*	8.3
05	M	13	*BBS1*	8.8
06	M	12	*BBS9*	7.9
07	F	15	*BBS10*	8.5
08*	F	13	*BBS1*	9.9
09	F	8	*BBS7*	10.6
10	M	9	*BBS1*	7.6
11	M	10	*BBS1*	7.6

*Second MRI scan not available

Table 8.2 Control group demographics

Participant number	***Gender***	***Age***	***Genotype***	***Time interval in months between assessments***
12	M	10	*BBS1*	6.9
13	F	8	*BBS7*	10.6
14	F	15	*BBS1*	10.3
15	M	11	*BBS10*	7.8
16	F	11	*BBS1*	8.1

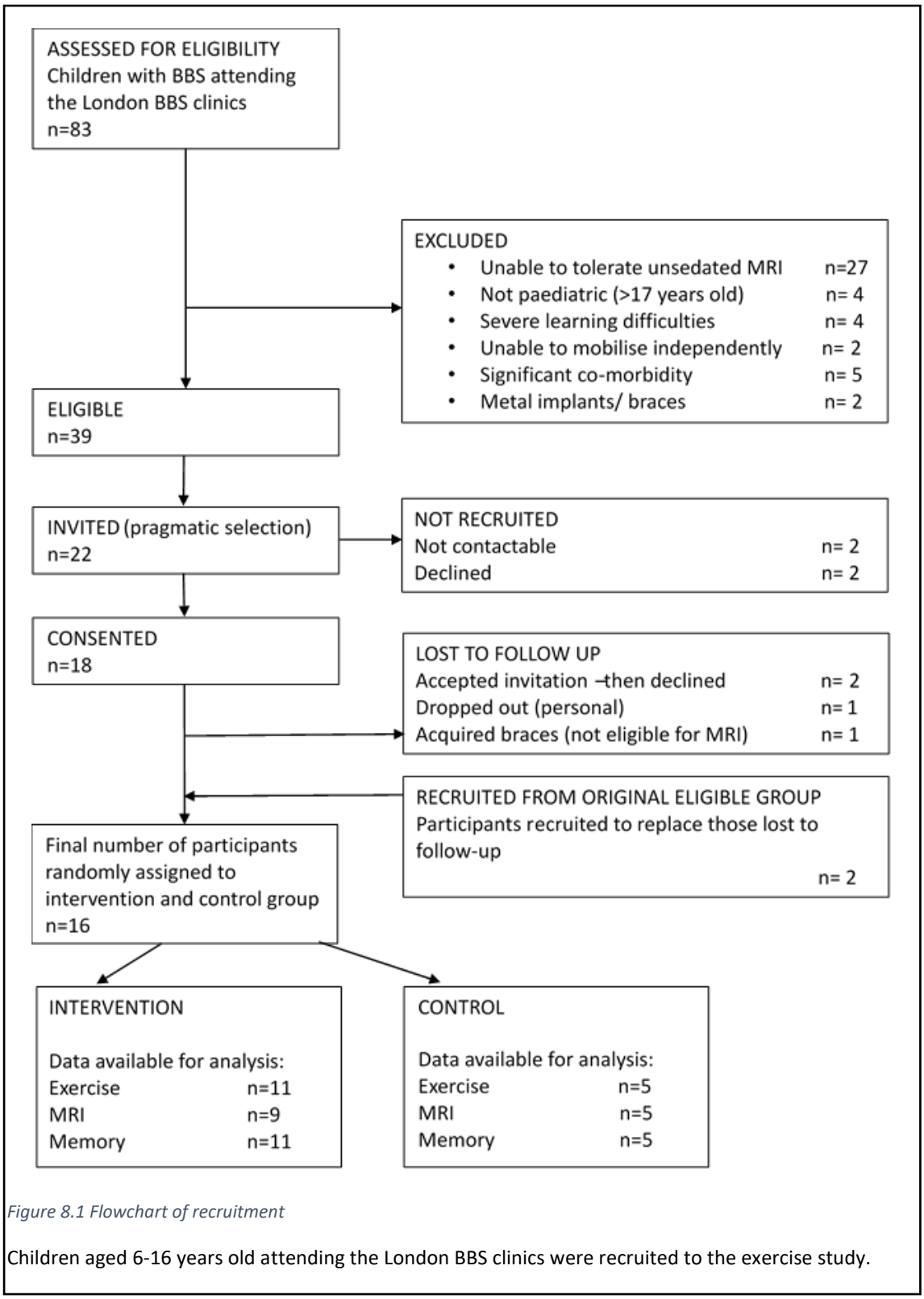


Figure 8.1 Flowchart of recruitment

Children aged 6-16 years old attending the London BBS clinics were recruited to the exercise study.

8.2.1 Physical activity outcomes

On comparison of performance at the first and second 10m-ISWT assessment the intervention group performed better than the control group. The intervention group

could run a median 76m (IQR: 89m) further at assessment 2 compared to assessment 1 whereas the control group could run a median 32m (IQR: 128m) further at assessment 2 (figure 8.2).

Level of change in intensity scores for each group were assessed. Those in the intervention group were able to perform at a higher intensity of exercise completing one further level, however this difference was not statistically significant (mean difference 1.0; 95%CI -1, 2; p=0.3). Both intervention and control groups had small increases in parental estimated levels ('exercise minutes gained') of activity post-intervention, but there was wide variability in reported activity levels (Table 8.3).

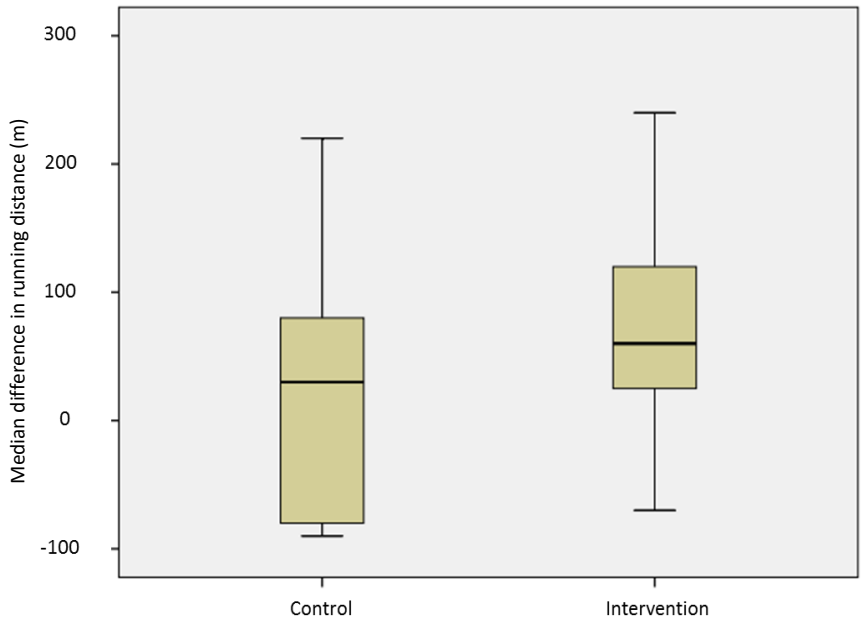


Figure 8.2 The intervention group was able to run further at the second assessment compared to baseline in comparison to the control group

Box and whisker plot demonstrates a higher median number of metres gained in the intervention group as well as considerable variability within and between the two groups. Figure courtesy of Dr Sean Ledger.

Table 8.3 Parental estimated exercise activity

	Intervention group	*Control group*
Pre-intervention Mean Estimated Activity (mins/week)	264	297
Post intervention Mean estimated Activity (mins/week)	276	303
Mean Change (mins/ week)	12	6

8.2.2 Imaging

8.2.2.1 Voxel based morphometry

The general linear model showed a significant time by group effect centred on the right posterior hippocampal region (T=5.59, xyz coordinates 36, -36, -9, cluster size 522, Family-wise error (FWE) corrected at cluster level: p=0.007), indicating greater local volume increase in the intervention group compared to the control group (Figure 8.3). At a lower statistical threshold (p<0.01) these changes also extend into the left hippocampal region and both clusters extended into the parahippocampal gyrus.

Regression of grey matter volume change (after small volume correction within a bilateral hippocampal region of interest) with change in 'exercise minutes gained' (parental report) resulted in significant positive association in the right (T=3.26, xyz coordinates 36, -17, -15, cluster size 260, p=.003) and left hippocampal region (T=2.47, xyz coordinates -30, -20, -23, cluster size 83, p=.014).

8.2.2.2 Diffusion

Localised differences in change in FA between the control and intervention groups were observed in the left hippocampus (figure 8.4a; p<0.01). Averaging the change in FA across the left hippocampus cluster (figure 8.4b) revealed a reduction in FA in the control group over the course of the investigation, but an increase in the intervention group. An increase in RD in the control group and decrease in RD in the intervention group was observed (figure 8.4c).

8.2.2.3 Perfusion

There was no significant difference in cerebral blood flow on comparison of global *ΔCBF* values between the control and intervention groups.

Linear regression analysis identified several significant correlations between regional change in cerebral blood flow (*ΔCBF*) values and the exercise variables. In the right hippocampus *ΔCBF* positively correlated with 'difference in distance' (R^2 = 0.28, p = 0.0499). ΔCBF values in the left and right post-central cortex correlated positively with 'exercise minutes gained'.

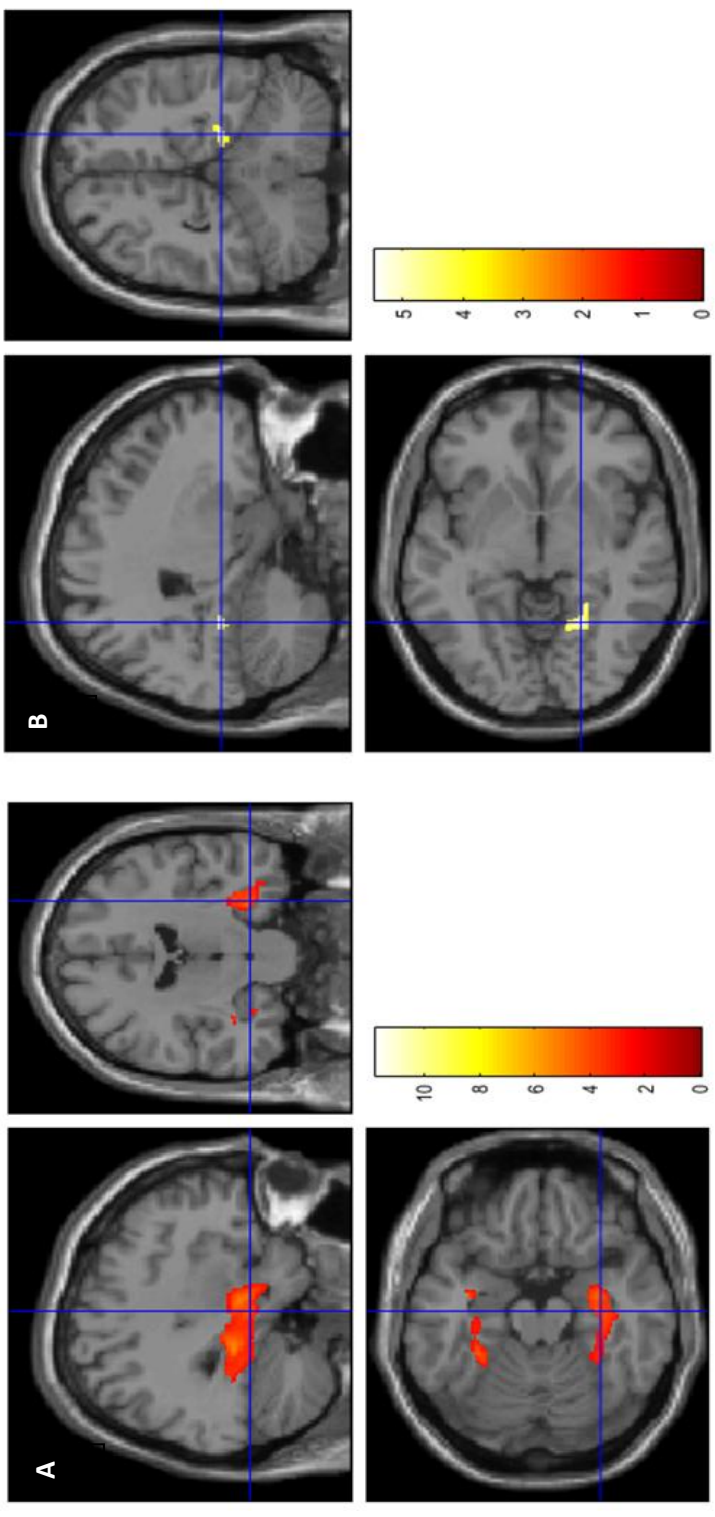


Figure 8.3. A greater hippocampal volume increase is observed in the intervention group compared to the control group.

A: Intervention group. B Control group. The general linear model demonstrates a significant time by group effect centred on the right hippocampal region (T=5.59, xyz coordinates 36, -36, -9, cluster size 522, FEW corrected at cluster level: p=0.01. A smaller cluster is also seen in the left hippocampal region. 8.2b: Control group. A small cluster is seen in the right hippocampus. Figure courtesy of Professor Torsten Baldeweg.

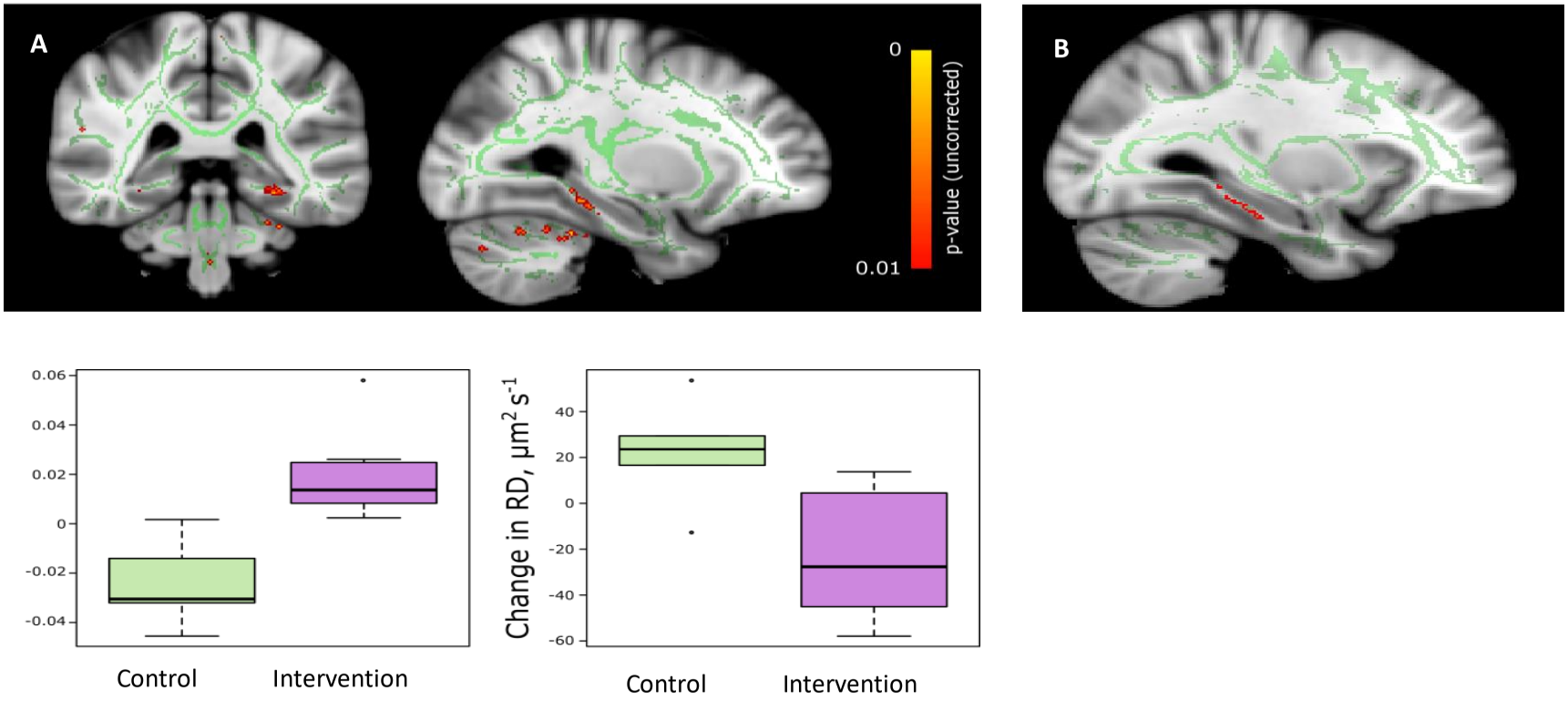


Figure 8.227 Changes in diffusion in the control versus intervention group demonstrating in increase in diffusion in the left hippocampus and left cerebellar peduncle in the intervention group

A-TBSS results showing group differences in the change in fractional anisotropy (FA) between time points. Red/yellow areas show a larger increase in FA in the intervention group than the control group ($p<0.01$, uncorrected). Results are overlaid onto an MNI T1w template. No significant difference was found in green voxels. B- Cluster near left hippocampus (shown in red) that is significantly different between control and intervention group. C- Boxplot showing change in FA (left) and Radial Diffusivity(RD) (right) between initial and final assessments for each group. The diffusion metrics were averaged over the significant cluster in close proximity to the left hippocampus. Figure courtesy of Dr Kiran Seunarine

8.2.3 Memory assessment

Both the control and intervention groups performed poorly on the memory 'Pair Games' test and had low learning scores (control group: M=2.4, SD=1.9, intervention group: M=2.3, SD=2.4)(figure 8.5a). These scores indicate that both groups learned on average less than three designs out of 10. Very few items were forgotten and the scores were close to 0 for both the control group (M=-0.6, SD=1) and the intervention group (M=-0.2, SD=1).

Mann-Whitney-U tests were run to determine if there were any differences between intervention and control groups on several measures. They did not significantly differ on measure of intellectual abilities, for either verbal intelligence quotient (mean difference -3.29; 95%CI -15.22, 8.6; p=0.510) or performance intelligence quotient (mean difference -6.12; 95%CI -21.38, 9.34; p=0.583). In addition, the two groups did not significantly differ from each other measures of learning (mean difference -0.09; 95%CI -2.43, 2.25; p=0.743) and forgetting (mean difference -0.42; 95%CI -1.69, 0.85; p=0.583) scores at baseline.

Change scores were calculated by subtracting performances at baseline from performance at time 2, for both learning and forgetting scores to provide measures of change from pre- to post-intervention and investigate the effect of exercise on cognitive performance. These scores are represented in figure 8.5b.

Change scores for the measure of learning were low for the control group (M=0.3, SD=1.1) and the intervention group (M=0.9, SD=1.7). Change scores for forgetting were low for the control group (M=-0.6, SD=1.8) and the intervention group (M=-0.09, SD=1.5).

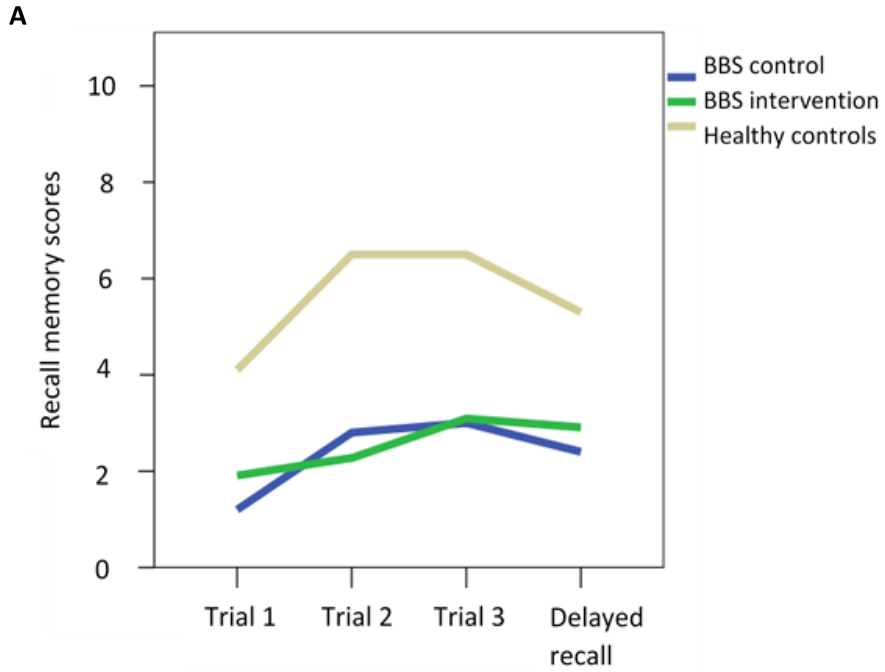


Figure 8.5 Memory testing in BBS

A: patients with BBS perform poorly in the memory test in comparison to healthy controls. Results here demonstrate a 'floor effect'- BBS patients struggle to complete the memory assessment in comparison to healthy controls. B: Performance in the 'pair games' recall test demonstrating improvement in learning change scores in the intervention group. Figure courtesy of Dr Sarah Buck.

8.2.4 Dietetics outcome

At the first assessment in the control group according to BMI-SDS classification one child was healthy, two overweight, one obese and one severely obese. At the second assessment four participants were overweight and one obese with one subject reducing their BMI-SDS by more than 0.25 in this time period.

In the intervention group at first assessment three children were classified as healthy, three overweight, one obese, three severely obese and one extremely obese. At the second assessment four were classified as healthy, two overweight, one obese, three severely obese and one severely obese with one subject reducing their BMI SDS by more than 0.25 in this time period.

8.3 Discussion

This proof of concept study demonstrates the positive correlation between exercise and hippocampal volume as well as white matter connectivity in individuals with BBS.

Brain volumetry: A significant volume increase was observed in both the left and right hippocampus with a greater effect demonstrated in the right hippocampus and maximal effect in the posterior end of the hippocampus. Chaddock-Heyman *et al*[324] reported global increased hippocampal blood flow in higher fit children with marginally greater blood flow in the posterior hippocampus. Killgore *et al* [307] studied healthy adults and found a positive correlation between the number of exercise minutes per week and right hippocampal tissue volume on voxel-wise analysis. Erickson *et al*[325] assessed the effect of physical activity on hippocampal volume in the elderly and assessed for a laterality effect but found that right and left hippocampi were equally affected by exercise. The

laterality seen here may be an effect of the small sample size or may be an, as yet, unreported true laterality effect of physical activity.

Cerebral diffusion: Diffusion tensor imaging findings support the hippocampal volume expansion identified on VBM.

Cerebral perfusion: Global cerebral blood flow changes did not identify any clear trends on examination of the control versus intervention group. However, regression analysis of fitness indicators identified some statistically significant local CBF changes in the right hippocampus and pre- and post-central cortex which may represent focal regions of angiogenesis as a result of increased physical activity. A previous study by Killgore *et al*[307] identified a positive correlation between hippocampal blood flow improvement and fitness change. This was particularly increased in the dentate gyrus which is considered to be the hub of hippocampal neurogenesis[307].

Memory: As a group, participants with BBS performed poorly in the memory test. A trend towards better performance in recall in the intervention group versus the control group was observed and provides encouraging preliminary data for a larger intervention trial. The majority of research on the relationship between memory, hippocampal volume and fitness has been conducted in the elderly and limited work on correlations in children. Chaddock *et al*[297] examined the effect of exercise on memory in pre-adolescent children and identified a correlation between hippocampal volume, maximal oxygen consumption (VO_{2max}) and relational (hippocampus specific) memory but not item memory (not hippocampus specific) supporting the findings we have identified in this trial.

Physical activity: Participants in the intervention group were able to run further and participated in more exercise minutes, as identified by parental diary reporting, than

those in the control group. Increased distance covered and intensity level achieved were small and not statistically significant. This may in part be due to the small sample sizes, but the clinical importance of these increases should be noted. Participants in the control group also appeared to increase their weekly exercise efforts during the study, but this is a well described phenomenon in other exercise trials[326]. It is possible that increasing the number of exercise sessions from once weekly and actively encouraging more general physical activity, would improve the exercise measures in the intervention group.

BMI: Early obesity is a primary feature of BBS; one-third of infants with a normal birth weight develop obesity by the age of one. A significant number of studies have reported health benefits with a reduction of 0.25 BMI SDS in obese children over a one year period with best results achieved in children under the age of 10[197,198]. One child in the control group and one child in the intervention group achieved greater than 0.25 BMI SDS reduction during the six month intervention. Hyperphagia and lower energy requirements due, in part, to reduced physical activity makes controlling rates of weight gain challenging in childhood and adolescence in patients in with BBS[184,185]. Maintenance of BMI SDS is therefore a significant clinical outcome in young people with this syndrome. In this study, individual BMI SDS change of -0.05 to +0.05 was considered maintenance. Participants in the intervention group achieved maintenance to a greater extent (73%) than participants in the control group (40%). This may indicate a correlation between the exercise intervention and greater energy expenditure and/or awareness of calorie ingestion.

Limitations The primary limitation of this study is the small number of participants. Although the change in hippocampal volume is extensive enough to be statistically

significant in such a small group, it allowed only for observations of trends in memory performance.

The small number of study participants may have affected results from MR imaging. It is notable that although changes in hippocampal volume were bilateral on VBM, diffusion tensor imaging identified more notable changes on the left. This is likely to be an artefact of the analysis process associated with the small number of participants.

Previous research has demonstrated a correlation between physical activity levels and hippocampal perfusion. This study did not replicate these findings. This may reflect a statistical error due to the sample size or it may be a result of the difficulties in accurately ascertaining perfusion in small volume structures. Alternatively, significant changes in perfusion may not be a pre-requisite for hippocampal volume change.

Accurately charting changes in fitness levels was a complex task in this study due to inherent challenges associated with working with children with learning difficulties. Whilst some of the participants were engaged and motivated to achieve to the maximum of their potential in the 10m-ISWT, others were unmotivated on one or both assessments hence skewing the outcome. Parent reported exercise diaries introduce significant recall bias and are inherently limited by the assumption that all forms of physical activity have the same potential to improve fitness.

The memory testing in this study was designed to entice children to engage as much as possible to optimise performance and complete tasks. The 'Pair games' test is designed primarily to assess hippocampus-specific recall memory, but also recognition memory which acts as an internal control. Additional benefit could potentially be ascertained through including a navigational memory test in light of the hypothesised visio-spatial function of the posterior hippocampus. A limitation of the memory testing in this study

is the requirement for prolonged concentration by participants which can be a challenge for young BBS patients. However, an advantage in testing young individuals with BBS is that most participants still have good visual function thus testing is not limited to auditory memory tests.

Future work This study demonstrated an impressive increase in hippocampal volume in participants in the exercise intervention. A bigger study is required to confirm the findings in this study and to ascertain if a statistically significant difference can be made in terms of a functional outcome as assessed by memory testing.

The study should be extended to include adult participants with BBS to ascertain if these results can be reproduced. Given the recent controversy concerning whether hippocampal neurogenesis is limited by age[327], such a study could add a valuable contribution to further our understanding as to whether this is possible.

In proceeding to a bigger study it would be prudent to employ accelerometers, which can accurately and objectively assess the frequency and level of physical activity achieved. This would overcome the inherent objectivity and confounding factors involved in parental reported exercise diaries and 10m-ISWT.

Other potential advances on the current intervention include a more structured exercise intervention with defined levels of progression. This would allow for more easy comparison between individuals and across groups. The disadvantage of introducing a structured programme would be the limitations this would place on fitness instructors' ability to be flexible in response to participants' variable learning difficulties, interests and wishes.

We observed some qualitative evidence for the positive effect of whole family involvement in an exercise intervention. Further studies may harness this effect when recruiting participants.

In addition to the improved hippocampal volume changes observed here, further studies will delineate the statistical significance of the trends towards improved hippocampal memory function as well as elucidating the positive effects of exercise on diffusion and perfusion to the brain.

Future work will ascertain the full effect of physical exercise on cognition, neuroplasticity and mood in patients with BBS. Independent of the outcomes in cognition this study demonstrates that patients with BBS are capable of participating in an exercise programme despite numerous physical challenges. This may not only benefit cognition but also weight and cardiovascular risk factors.

This study demonstrated that although patients with BBS are at a genetically pre-determined disadvantage with respect to hippocampal volume[295] their potential for neurogenesis in the hippocampus remains intact and can be harnessed through a weekly supervised exercise programme. In the future this could identify a pathway to improved neurocognition in forms of intellectual disability.

9 Conclusion

For my PhD I aimed to understand BBS in all its guises, how it progresses from birth to death, the enigma of how this condition can present with such variability, and deciphering which elements of the condition really matter to the patients. My ambition was to prepare BBS for the next era in clinical genomics- therapies for rare diseases.

My starting point was the concerns and worries voiced in clinics by patients and carers, primarily addressing renal disease, rod-cone dystrophy, obesity, cardiometabolic disease, learning and memory as well as future therapies- both in the form of lifestyle modifications and pharmaceutical intervention.

One of the major challenges of studying a rare disease is the lack of a representative cohort, primarily due to the difficulty in identifying a big group of patients. Since their inception in 2010, the UK national BBS clinics have grown to serve more than 500 patients, representing the single biggest reported cohort worldwide. This has not only served to train clinicians to be true experts in the condition but also provides a unique population to study phenotype and natural history.

9.1 What we know now

Before I started my thesis I was unable to provide much reassurance for parents, carers and patients about the likelihood of developing severe renal disease as well as related comorbidities. Very little was known about this aspect of the condition other than that it was a significant contributor to mortality in this patient group. Following the renal study (chapter 3), which included 350 patients across all four sites, we are now able to reassure patients that end stage renal disease is relatively uncommon, affecting around 6-8% of patients, and that mild to moderate renal disease can be managed with lifestyle

and pharmacological intervention. Furthermore, for those patients who have the common *BBS1* p.Met390Arg genotype and have reached adulthood with no functional or structural renal deficit the chance of developing severe renal disease is almost negligible. Since the first (and thus far only) study on mortality and BBS was published in 1996[204], where the recorded cause of death was renal disease in approximately half the patients, healthcare systems worldwide have progressed to delivering a more equitable renal transplant service for all patients, including those with complex rare diseases. It is likely that one can extrapolate from this that death due to end stage renal disease in BBS is now a rare event and no death from renal disease has been observed in the UK national BBS.

Blindness as a consequence of rod-cone dystrophy inevitably ensues and is one of the most feared aspects of BBS. Following the ophthalmology study (chapter 4) it is now possible to give patients some guidance on visual deterioration based on genotype at the population level. Although it is not possible to predict the time course of visual deterioration at the level of the individual, this provides useful pointers for patients which can help with forward planning, in particular in regard to learning the necessary skills required for daily living with visual impairment. The suspected hypomorphic phenotype observed in patients with the *BBS1* p.Met390Arg genotype is reinforced. The study also calls into question whether it is time to revise the diagnostic criteria. One possibility is to incorporate molecular confirmation into the diagnostic criteria or, alternatively, to consider the lack of ophthalmological evidence of rod-cone dystrophy as a sign that an alternative diagnosis should be sought.

The obesity associated with BBS is an often underestimated burden of the disease (chapter 5). Although the obesity is amenable to traditional weight loss strategies, often the willpower required to resist the appetite signalling disruption as well as the magnitude of the task is too overwhelming for many patients. Understanding the extent of obesity as well as the natural history in this patient population is paramount to mapping out the cardiometabolic risks as well as assessing the requirement for pharmacological and surgical intervention in this population group. For children, the mean obesity level at first clinic visit was within the severe obesity range (BMI-SDS >+2.67), dropping below this point at the third clinic visit but with mean BMI remaining in the obese range. Variation in BMI-SDS was high -especially in children under the age of 4. Statistically significant reductions in weight loss were observed on successive clinic visits and further work assessing the effect on markers of cardiometabolic health may unveil the effect this has on morbidity risks. Adults did not attain statistically significant reductions in weight loss on successive clinic attendances but a small mean weight loss was achieved and sustained on successive visits. This is an advance on the expected age-related weight increase correlating with age observed in both this patient group as well as the general population. Children with mutations in *BBS10* were statistically more likely to have a higher BMI-SDS than children with mutations in *BBS1* in line with the genotype-phenotype correlations observed in other aspects of BBS. This difference in genotype and BMI did not reach statistical significance in the adult population and was possibly affected by the mean older age of patients with *BBS1* mutations.

The discrepancy in BMI reduction in the adult and child populations may reflect a number of factors including the intensity of dietetics intervention, ability of parents/ guardians to control the food environment as well as growth metabolic factors which

play to the advantage of children. However, for both groups BMI/BMI-SDS remains at an unacceptably high level, which is likely to affect future cardiometabolic risk. Pharmacological intervention to reduce the level of obesity in patients with BBS remains an attractive option. Novel therapies such as Setmelanotide which intercept in the MC4 pathway, which is predicted to be affected in BBS, are an attractive option. The emergence of repurposed drugs such as the GLP-1 agonists which have been found to be effective in patients with MC4 receptor mutations are also an exciting possibility.

A question that remains to be answered in BBS is whether the condition is accompanied by a shorter life expectancy. Given the small number of affected individuals this is inherently difficult to answer. Life span does not appear to be dramatically reduced, as very few deaths have occurred in the population observed in the national BBS clinics since inception in 2010. Previous research points to cardiometabolic disease as a primary cause of death. The cardiovascular risk factor study (chapter 6) attempted to identify if genotype and mutation type were determinants of higher cardiometabolic risk and identified patients with two missense mutations in *BBS1* as being at the lowest risk. Viewed in the context of the obesity study, an increased index of concern is prudent in assessing and managing the cardiometabolic health in all patients with BBS but in particular in those who don't have two missense mutations in *BBS1*.

Identifying a cellular phenotype in BBS (Chapter 7) would be a significant step forward in preparing for future pharmacological intervention. Working with primary cell cultures inherently introduces variability but also allows for the identification of a biologically relevant phenotype. On average, fibroblasts from individuals with mutations in *BBS10* proliferated faster than fibroblasts from patients with mutations in *BBS1* and control fibroblasts. No clear structural cilia phenotype was identified in this study except in

CEP290 and significant variability between samples from different individuals was observed. A major obstacle is the inability to study the phenotype and natural history of retinal degeneration in BBS. Not only is the eye phenotype consistently present, but since photoreceptors are intact at birth in BBS, there is a window of opportunity for therapeutic intervention in the BBS eye phenotype as opposed to the kidney where structural aberrations are usually present at birth. Our evolving ability to transform pluripotent stem cells into specific tissues and organ systems *ex vivo,* such as retinal cells and eye balls, is likely to allow a deeper understanding of the BBS cellular phenotype.

Management of BBS so far has been largely symptomatic with no targeted therapy. The potential effect of lifestyle on the expression of genetic diseases is only beginning to come to light. Lifestyle has previously been largely overlooked as a significant intervention to improve functional outcomes. The exercise study (chapter 8) demonstrated that hippocampal volume can be improved by physical exercise even in individuals who have a genetically determined smaller volume hippocampus. Future work will hopefully determine whether this has a measurable functional effect on memory, which could contribute substantially to the quality of life of individuals living with BBS. Anecdotal evidence from the UK national BBS clinics suggests that the individuals with the best quality of life are those who are able to live independently. This is most likely to be achieved when individuals have learned independent living skills in late childhood or early adulthood when many patients still have some remaining visual function. Improved recall memory could contribute by improving the efficiency by which these skills are retained and therefore, by inference, not only improve school performance but also quality of life.

When I see patients in the BBS clinics now, I am able to provide a degree of reassurance about the likelihood of developing kidney disease, realistic expectations about the decline of visual function, insight into how obesity is likely to progress, counsel patients and carers about the increased cardiovascular risks and multiple beneficial effects of physical activity. All the evidence suggests that patients with mutations in *BBS1,* in particular those who are homozygous for the common p.Met390Arg missense mutation, have a milder phenotype than patients with other genotypes and mutation types. To an extent this is reflected in the fibroblast cellular phenotype, where fibroblasts from patients with *BBS10* mutations are more likely to display aberrant proliferation compared to controls and *BBS1* cells, albeit with significant variability, mirroring the variability in phenotype seen in the clinics.

9.2 What the future holds

Our understanding of the BBS phenotype and natural history has increased exponentially in the last ten years. Key to taking BBS into the next era of genomic therapies is the identification of research priorities as well as disrupting the process by which drug therapies are currently developed for rare diseases to create a more streamlined, cost effective approach.

Traditionally research priorities have been set by researchers and funders, leading to a biased priority agenda. Increasingly, however, the value of patient and public involvement is being recognised. Organisations such as the James Lind Alliance bring together patients, carers and clinicians to create a formalised structure for research priorities which not only brings the needs of the patients to the centre, but also delivers a formalised structure for research priority setting which is more likely to generate

funding[328]. Patients and families are increasingly being recognised as key stakeholders in the process of developing novel drugs. Not only are they highly qualified in pinpointing aspects of a disease which most significantly affects quality of life, they can also advise on functionally useful trial end-points and assessing risk/ benefit profiles[329].

As with 95% of rare diseases[330], not a single FDA approved drug exists to treat BBS. Historically, rare diseases have not garnered much interest or investment from the pharmaceutical industry due to the cost of developing new drugs and the low number of potential beneficiaries. Other obstacles include the difficulty in accessing potential clinical trial participants due to low prevalence and consequent requirements for multinational studies which can present logistical hurdles. These challenges can be discouraging to the pharmaceutical industry in light of limited financial reward. This does, however, appear to be changing. Not only can understanding aspects of rare diseases, where pathogenic pathways are often more easily identifiable, tell us a great deal about the pathogenic process in common diseases, but it is also possible that the pathway to developing therapies for rare diseases can be simplified and streamlined to a greater extent than it is today. For instance, Ekins (2017)[330] suggests that the current challenges in developing efficient production lines for gene therapy for rare diseases consist of the lack of shared and open collaboration in identifying good quality vectors and patents. Ekins suggests that a unified, industrialised approach, whereby expertise is concentrated in a limited number of geographical locations where groups of experts work on specific steps in the development process would be a more efficient approach[330]. This is an enticing possibility that may require substantial funding incentives to become a reality.

This PhD began with a view to developing a more sophisticated understanding of BBS and bringing those affected closer to therapies to alleviate some of the challenges they face. I hope this has, at least in part, been achieved.

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Appendix 1. Complete list of fibroblast cell cultures established

Code	Gene	Mutation 1	Mutation 2
RT1	*BBS2*	p.Tyr24*	p.Leu168Phefs*33
RT2	*BBS2*	p.Arg189*	p.Arg189*
CO1	*BBS10*	p.Glu19*	p.Tyr197Cys
CO2	*BBS1*	p.Met390Arg	p.Tyr284Serfs*5
CO3	*BBS1*	p.Met390Arg	c.47+8C>T
CO4	*BBS1*	p.Met390Arg	p.Met390Arg
CO5	*BBS1*	p.Met390Arg	p.Met390Arg
CO6	*BBS1*	p.Met390Arg	p.Met390Arg
CO8	*BBS1*	p.Met390Arg	p.Met347Argfs*27
CO7	*BBS9*	p.Lys626Argfs*22	Het
CO9	*BBS1*	p.Met390Arg	p.Met390Arg
CO10	*BBS1*	c.723+1G>A	p.Met390Arg
CO11	*BBS1*	p.Met390Arg	p.Met390Arg
GST-01	*BBS1*	p.Met390Arg	p.Met390Arg
GST-02	Genotype unknown		
GST-03	*BBS2*	p.Arg275*	p.Val429Glyfs*44
GST-04	Genotype unknown		
GST-05	*BBS1*	p.Met390Arg	p.Met390Arg
GST-06	Genotype unknown		
GST-07	*BBS1*	p.Met390Arg	p.Met390Arg
GST-08	*BBS1*	p.Met390Arg	p.Met390Arg
GST-09	*BBS2*	p.Arg413*	p.Arg413*
GST-10	*BBS1*	p.Met390Arg	p.Met390Arg
GST-11	*BBS12*	p.Phe473del	p.Phe473del
GST-12	*BBS10*	p.Asp102Glufs*6	p.His300Pro
GST-13	*BBS1*	p.Met390Arg	p.Met390Arg
GST-14	*BBS2*	p.Asp104Ala	p.Arg632Pro
GST-15	*BBS1*	p.Tyr284Serfs*5	p.Met390Arg
GST-16	*BBS1*	p.Tyr284Serfs*5	p.Met390Arg
GST-17	*BBS1*	p.Tyr284Serfs*5	p.Met390Arg
GST-18	*BBS1*	p.Met390Arg	p.Met390Arg
GST-19	*BBS1*	p.Met390Arg	p.Gly73*

GST-20	*BBS10*	p.Arg95Ser	p.Val707*
GST-21	*BBS2*	p.Asp104Ala	p.Arg632Pro
GST-22	*BBS1*	p.Met390Arg	p.Met390Arg
GST23	*BBS2*	p.His399Glnfs18	c.2060-1G>T
GST24	*BBS10*	p.Thr79Asnfs*17	p.Val330Ala
GST25	*BBS10*	c.530A>G	c.530A>G
GST26	Genotype unknown		
GST27	*BBS1*	p.Met390Arg	p.Met390Arg
GST28	*BBS10*	p.Cys91Leufs*5	p.Cys91Leufs*5
GST29	*BBS1*	p.Met390Arg	p.Gly73*
GST30	*BBS1*	p.Met390Arg	p.Gly73*
GST32	*BBS12*	c.1589T>C	c.1589T>C
GST33	*BBS1*	p.Met390Arg	p.Met390Arg
GST34	*BBS2*	p.Leu260_lle261insMe t	p.His525Phefs*21
GST35	*BBS1*	p.Met390Arg	p.Met390Arg
GST36	*BBS1*	p.Arg570*	p.Arg570*
GST37	*BBS1*	p.Arg570*	p.Arg570*
GST38	*BBS1*	p.Arg570*	p.Arg570*
GOSH1	*CEP290/BBS14*	p.Met1723Val	c.-1003T>C/ 12:88142875
GOSH2	*BBS2*	p.Tyr24*	p.Arg275*

Appendix 2: Prizes and presentations

Prizes

- Winner 3 Minute Thesis for UCL 2018. https://www.ucl.ac.uk/child-health/file/9135
- MRC Max Perutz Public engagement prize Shortlisted candidate. 2014

Oral communications

- Forsythe E. The secret to a bigger, better brain'. Great Ormond Street Institute of Child Health Open day. November 2018.
- Forsythe E, Sparks K, Seunarine K, Hales P, Buck S, Clegg H, Lin ML, Flack S, Ledger S, Harniess P, Clark C, Beales P, Baldeweg T. The surprising lifestyle intervention that facilitates brain neuroplasticity in Bardet-Biedl syndrome. British Society for Genomic Medicine, October 2018
- Forsythe E, Sparks K, Best S, Borrows S, Hoskins B, Sabir A, Barrett T, Williams D, Mohammed S, Goldsmith D, Milford DV, Bockenhauer D, Foggensteiner L, Beales PL. 'Renal disease in Bardet-Biedl syndrome', Nephrology week, UCL Great Ormond Street Institute of Child Health, April 2018.
- Forsythe E, Sparks K, Best S, Borrows S, Hoskins B, Sabir A, Barrett T, Williams D, Mohammed S, Goldsmith D, Milford DV, Bockenhauer D, Foggensteiner L, Beales PL.' Renal disease in Bardet-Biedl syndrome' CME Course: Multidisciplinary management of inherited kidney diseases. Fundacio Puigvert, Barcelona, Spain, Sept 2017
- Forsythe E, Sparks K, Best S, Borrows S, Hoskins B, Sabir A, Barrett T, Williams D, Mohammed S, Goldsmith D, Milford DV, Bockenhauer D, Foggensteiner L, Beales PL. 'Predictors of renal disease in Bardet-Biedl syndrome'. British Society for Genomic Medicine annual conference 2016.
- Forsythe E, Sparks K, Best S, Borrows S, Hoskins B, Sabir A, Barrett T, Williams D, Mohammed S, Goldsmith D, Milford DV, Bockenhauer D, Foggensteiner L, Beales PL.

Risk Stratification in Bardet-Biedl syndrome. British Society for Genomic Medicine annual conference 2014, invited speaker.

Public Engagement

- About my research for donors to Great Ormond Street Hospital Children's Charity (2015). http://www.gosh.org/get-involved/philanthropy/bringing-research-life/impact/news/bardet-biedl-syndrome
- Great Ormond Street Hospital Annual Symposium on Research and Innovation (video-2015). http://www.gosh.nhs.uk/research-and-innovation/nihr-great-ormond-street-brc/brc-video-library/brc-annual-symposium-great-expectations-improving-care-bardet-biedl-syndrome
- Invited guest blogger on 'Me & My Genes'. "Cilia: Sense and Sightability. A UCL initiative, this is a family friendly blog targeted at individuals with limited prior knowledge of genetics. http://blogs.ucl.ac.uk/clinical-molecular-genetics/2014/12/08/cilia-sense-and-sightability/

Appendix 4: First author publication: Genetic predictors of cardiovascular morbidity in Bardet-Biedl syndrome.

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Short Report

Genetic predictors of cardiovascular morbidity in Bardet–Biedl syndrome

Forsythe E., Sparks K., Hoskins B.E., Bagkeris E., McGowan B.M., Carroll P.V., Huda M.S.B., Mujahid S., Peters C., Barrett T., Mohammed S., Beales P.L. Genetic predictors of cardiovascular morbidity in Bardet–Biedl syndrome.
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Bardet–Biedl syndrome is a rare ciliopathy characterized by retinal dystrophy, obesity, intellectual disability, polydactyly, hypogonadism and renal impairment. Patients are at high risk of cardiovascular disease. Mutations in *BBS1* and *BBS10* account for more than half of those with molecular confirmation of the diagnosis. To elucidate genotype–phenotype correlations with respect to cardiovascular risk indicators 50 patients with mutations in *BBS1* were compared with 19 patients harbouring *BBS10* mutations. All patients had truncating, missense or compound missense/truncating mutations. The effect of genotype and mutation type was analysed. C-reactive protein was higher in those with mutations in *BBS10* and homozygous truncating mutations ($p = 0.013$ and $p = 0.002$, respectively). Patients with mutations in *BBS10* had higher levels of C peptide than those with mutations in *BBS1* ($p = 0.043$). Triglyceride levels were significantly elevated in patients with homozygous truncating mutations ($p = 0.048$). Gamma glutamyl transferase was higher in patients with homozygous truncating mutations ($p = 0.007$) and heterozygous missense and truncating mutations ($p = 0.002$) than those with homozygous missense mutations. The results are compared with clinical cardiovascular risk factors. Patients with missense mutations in *BBS1* have lower biochemical cardiovascular disease markers compared with patients with *BBS10* and other *BBS1* mutations. This could contribute to stratification of the clinical service.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Key words: Bardet–Biedl syndrome – cardiovascular morbidity – genotype–phenotype correlation – mutation type

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Bardet–Biedl syndrome (BBS) is a pleiotropic autosomal recessive ciliopathy characterized by retinal dystrophy, post-axial polydactyly, obesity, learning difficulties, hypogonadism and renal dysfunction (1, 2). There is a high prevalence of cardiovascular, endocrine and renal disorders among patients with BBS (3). In order to optimize the clinical management, it is imperative to identify patients who are most at risk of disease-associated morbidity and mortality.

Since the clinical criteria for a diagnosis were proposed, 19 genes (*BBS1–BBS19*) have been discovered (4, 5). BBS genes code for proteins that localize to the basal body of the cilium. Mutations lead to defective cilia accounting in part for the variable effects observed in BBS. A clinical diagnosis can be confirmed by sequencing the known disease-causing genes in 80% of patients (authors' own unpublished data).

The majority of pathogenic mutations are found in *BBS1* and *BBS10* accounting for 23.2% and 20%, respectively in populations of northern European descent (4). The commonest mutation is M390R found in 82.5% of a cohort of British patients with *BBS1* mutations (4). The frameshift mutation C91LfsX5 is prevalent in patient populations with *BBS10* mutations.

Variable phenotypic expressivity is a hallmark of BBS, however, even among patients with the same genotype, interfamilial and intrafamilial phenotypic variability is common. Mutations in other BBS genes may modify the phenotype, accounting for this variability (6). Several studies have attempted to identify a genotype–phenotype correlation in BBS (7–10). These have primarily focused on physical features and have been limited by small sample sizes or participants from the same kindred.

This is the first study to explore the correlation between genotype, mutation type and morbidity in BBS. We examine indicators of cardiovascular, metabolic and renal morbidity in a large cohort of patients with BBS and compare the two most commonly mutated genes: *BBS1* and *BBS10*.

Methods

Patients

Two hundred and thirty nine patients attending the national Bardet-Biedl Syndrome clinics in London and Birmingham were assessed for height, weight, blood pressure, BBS mutation analysis, full blood count, renal function, liver function, inflammatory markers, endocrine and lipid profile. Information on cardiovascular risk factors was collected retrospectively from patient notes. The following clinical parameters were ascertained: (i) hypertension (defined as a blood pressure over 140/90 or normotensive requiring antihypertensive medication), (ii) hypercholesterolaemia requiring hypolipidaemic agents, (iii) diabetes mellitus requiring hypoglycaemic medication, (iv) structural renal abnormalities and/or dialysis or renal transplant, and (v) structural cardiac abnormalities. Patients were predominantly of Caucasian origin. Referrals were made primarily via the British national patient support group and clinical geneticists in the United Kingdom.

Mutation screening

Eighty four of the 239 patients had two known pathogenic mutations in BBS genes. Of these, 73 harboured two mutations in *BBS1* or *BBS10*. Mutation analysis was primarily undertaken through targeted sequencing of the four most common mutations: M390R in *BBS1*, and Y24X and R275X in *BBS2* and C91LfsX5 in *BBS10*. Where only one mutation was found, full sequencing of the relevant gene was performed to identify a second mutation.

Statistical analysis

Analysis was targeted to *BBS1* and *BBS10* patients with truncating and/or missense mutations to allow for adequate sample sizes.

We applied a two pronged approach to statistical analysis. Mann–Whitney *U* test, Kruskal–Wallis test and analysis of variance (ANOVA) were performed as appropriate to identify associations between genes (*BBS1 vs BBS10*) and mutation types (homozygous truncating mutations, heterozygous missense and truncating mutations or homozygous missense mutations). Multivariable linear regression analysis was applied to variables which were statistically significant on univariable analysis and/or known indicators of metabolic, renal and cardiovascular disease. Statistical analyses were carried out using SPSS version 21.0 (SPSS Inc., Chicago IL).

Results

Distribution of patients

Fifty two patients harboured two mutations in *BBS1* and 21 harboured two mutations in *BBS10*. This included nine pairs and two sets of three siblings. DNA results were classified according to gene and mutation. Mutation type was further classified according to the predicted severity. Most patients had either homozygous missense mutations, homozygous truncating mutations or a heterozygous missense and truncating mutations. Two patients with *BBS1* mutations and two patients with *BBS10* mutations harboured splice site mutations and were excluded from the statistical analysis. Figure 1 demonstrates the distribution of mutation types in the *BBS1* and *BBS10* genotypes analysed in this study, illustrating the higher proportions of missense mutations in *BBS1* and truncating mutations in *BBS10*.

Of the remaining 69 patients seen in the clinic with a mutation in *BBS1* or *BBS10* the mean age was 28.25 (SD: 14.41, range: 0–59). The mean (SD) age of patients with a *BBS1* mutation was 30.5 (15.6) years. In contrast the mean age (SD) of patients with a mutation in *BBS10* was 22.32 (8.95) years ($p = 0.034$). Figure 2 illustrates the age distribution of patients with *BBS1* and *BBS10* included in this study. Twenty four (48%) patients with a mutation in *BBS1* were female and 26 (52%) were male. Twelve (63.2%) patients with mutations in *BBS10* were female and seven (36.8%) were male.

Clinical parameters

Genotype–phenotype associations were tested for all clinical variables. Univariable analysis comparing patients with *BBS1 vs BBS10* demonstrated a statistically significant difference in age, height,

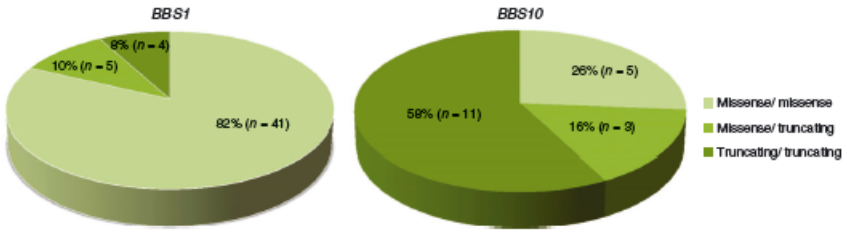

Fig. 1. Distribution of mutation type in patients with *BBS1* and *BBS10* included in the analysis.

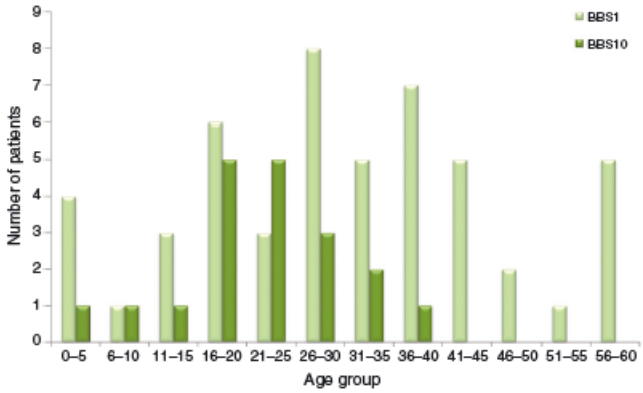

Fig. 2. Age distribution of patients with mutations in *BBS1* and *BBS10* included in the analysis.

Table 1. Genotype–phenotype correlation: univariable comparison of statistically significant parameters. Patients with *BBS1 vs BBS10*. Only statistically significant results are included. The full analysis is available in Tables S1, Supporting information

	BBS 1		BBS 10		
	Mean	SD	Mean	SD	p-Value[a]
Anthropomorphic measurements					
Age at clinic	30.5	−15.48	22.32	−8.95	0.034
Height (cm)	163.92	−28.17	162.93	−9.31	0.031
Inflammatory markers					
CRP (mg/l)	5.69	−2.74	9.53	−7.12	0.04
Endocrine profile					
C peptide (ng/ml)	1295.88	−740.13	2333.3	−1501.27	0.014
Lipid profile					
Triglycerides (mmol/l)	1.5	−0.73	1.98	−0.94	0.049
Renal profile					
Potassium (mmol/l)	4.16	−0.48	4.38	−0.42	0.015
Albumin/creatinine ratio	7.1	−22.39	5.3	−11.23	0.032

ANOVA, analysis of variance; BBS, Bardet–Biedl syndrome, CRP, C-reactive protein.
[a]p-Value obtained from ANOVA test or Mann–Whitney *U* test.

Table 2. Mutation type-phenotype comparison: univariable comparison of statistically significant parameters. Homozygous missense; heterozygous truncating and missense and homozygous truncating mutations. Only statistically significant results are included. The full analysis is available in Table S2

	Missense/missense		Missense/truncating		Truncating/truncating		
	Mean	SD	Mean	SD	Mean	SD	p-Value[a]
Lipid profile							
HDL cholesterol (mmol/l)	1.28	−0.25	1.09	−0.12	1.1	−0.23	0.022
Liver profile							
Gamma glutamyl transferase (U/l)	29.21	−16.22	70.33	−10.02	62.75	−40.01	0.027

HDL, high-density lipoprotein.
[a]p-value obtained from ANOVA test or Kruskal–Wallis test.

C-reactive protein (CRP), c-peptide, triglycerides, potassium, and albumin–creatinine ratio (Table 1). Comparison of mutation types revealed a statistically significant difference in high density lipoprotein (HDL) cholesterol and gamma glutamyl transferase (GGT) (Table 2).

We applied multivariable analysis to selected variables based on association with cardiovascular risk and controlled for confounding factors. Statistically significant results are displayed in Tables 3 and 4 and discussed here.

Inflammatory markers

A statistically significant difference in CRP is observed. Patients with a mutation in *BBS10* or homozygous truncating mutations have a significantly higher CRP ($p=0.013$ and $p=0.002$, respectively). Analysis of white cell count and other blood count parameters did not reach statistical significance.

C peptide

C peptide levels were significantly higher in patients with *BBS10* compared with *BBS1* mutations ($p=0.043$).

Lipid profile

Triglycerides levels were significantly higher in patients with homozygous truncating mutations ($p=0.048$) than those with other mutation types.

Liver function

Multivariable analysis demonstrated significantly higher GGT in patients with homozygous truncating or heterozygous missense and truncating mutations than those with homozygous missense mutations ($p=0.007$ and $p=0.002$, respectively).

Clinical cardiovascular risk factors

Sixty seven patients had a full lipid profile, of whom 14 had hypercholesterolaemia. Hypertension was identified in 23 of 65 patients. Fifteen of 69 patients had a diagnosis of diabetes mellitus. Five of 69 patients had the results of an echocardiogram documented. Of these, one had an innocent murmur, two had a ventricular septal defect, one had an atrioventricular septal defect and one patient had aortic valve stenosis. All of these patients had two missense mutations in *BBS1*. Twenty nine patients had renal ultrasounds. Sixteen of these had abnormal results ranging from benign structural malformations to sonographic evidence of chronic renal failure. Two patients had renal transplants and one was on dialysis, all of whom had two truncating mutations in *BBS10*. Figure 3a,b illustrate the prevalence of the clinical cardiovascular risk factors. Cardiac abnormalities are not included in these diagrams as only five patients had documented echocardiograms and we therefore perceive the results not be representative of the group as a whole.

Table 3. Genotype–phenotype comparison: multivariable comparison of selected parameters found to be statistically significant. *BBS1 vs BBS10*. Only statistically significant results are included in this table. The full analysis is available in Table S3

	β estimate	95.0% CI	p-Value[a]
CRP (mg/l)			
Genotype			
BBS1	Reference	–	–
BBS10	**4.08**	**(0.90, 7.25)**	**0.013**
Age	0.06	(−0.05, 0.18)	0.295
BMI	0.1	(−0.08, 0.29)	0.266
C peptide (ng/ml)			
Genotype			
BBS1	Reference	–	–
BBS10	**942.94**	**(32.26, 1853.61)**	**0.043**
BMI	−4.86	(−67.84, 58.12)	0.876
Blood glucose	92.08	(−71.24, 255.41)	0.258

BBS, Bardet–Biedl syndrome; BMI, body mass index; CI, confidence interval; CRP, C-reactive protein. Significant values are highlighted in bold.
[a]p-value obtained from linear regression model.

Discussion

To our knowledge this is the only study comparing cardiovascular risk factors for patients with *BBS1* and *BBS10* – the two most commonly mutated genes in patients from Europe and North America. It is the first published study to compare the BBS phenotype according to mutation type. Several studies have suggested that

Table 4. Mutation type-phenotype comparison: multivariable comparison of selected parameters found to be statistically significant. Homozygous missense; heterozygous truncating and missense and homozygous truncating. Only statistically significant results are included in this table. The full analysis is available as Table S4

	β estimate	95.0% CI	p-Value[a]
CRP (mg/l)			
Mutation type			
Missense/missense	Reference	–	–
Missense/truncating	−0.65	(−4.42, 3.12)	0.729
Truncating/truncating	**5.33**	**(1.99, 8.68)**	**0.002**
Age	0.06	(−0.05, 0.17)	0.272
BMI	0.14	(−0.03, 0.31)	0.11
Triglycerides (mmol/l)			
Mutation type			
Missense/missense	Reference	–	–
Missense/truncating	0	(−0.67, 0.67)	0.998
Truncating/truncating	**0.56**	**(0.01, 1.11)**	**0.048**
Gender			
Female	Reference	–	–
Male	**0.52**	**(0.07, 0.98)**	**0.026**
BMI	**0.03**	**(0.00, 0.06)**	**0.05**
Age	−0.01	(−0.03, 0.01)	0.452
Gamma glutamyl transferase (U/l)			
Mutation type			
Missense/missense	Reference	–	–
Missense/truncating	**44.22**	**(17.90, 70.54)**	**0.002**
Truncating/truncating	**29.32**	**(8.72, 49.91)**	**0.007**
Gender			
Female	Reference	–	–
Male	**17.94**	**(4.12, 31.76)**	**0.013**
BMI	**1.05**	**(0.15,1.95)**	**0.025**
Age	0.24	(−0.28, 0.76)	0.349

Significant values are highlighted in bold.
[a]p-Value obtained from linear regression model.

there is little evidence of a genotype–phenotype correlation in BBS and proposed that this may be because BBS proteins contribute to a common molecular pathway (4). However, there is an emerging evidence of some genotype–phenotype correlations (7, 10). Feuillian et al. (10) reported that patients with *BBS1* had lower insulin resistance compared with patients with *BBS10*. Observations from the British nationally commissioned clinic suggest that patients with two mutations in *BBS10* are often more severely affected than those with *BBS1* although there is considerable variation.

CRP increases with obesity and inflammation. Chronically raised CRP indicates a higher risk of cardiovascular morbidity (11). This study shows that patients with *BBS10* genotypes and/or two truncating mutations have a significantly worse CRP value. As CRP is a physiological marker of inflammation and infection, it is notable that there was no statistically significant difference in white cell count or weight between patients with different genes or mutation types. This suggests that patients with missense mutations in *BBS1* may be at lower risk of cardiovascular disease than patients with *BBS10* or other mutations in *BBS1*.

C peptide is used as a marker of insulin resistance but has in recent years been recognized as an independent bioactive peptide exerting effects on microvascular function, correlating with macrovascular complications and cardiovascular death (12). Our results demonstrate that patients with mutations in *BBS10* have significantly higher levels of C-peptide indicating insulin resistance, supporting the suggestion that they are at higher risk of cardiovascular disease than patients with mutations in *BBS1*.

Raised triglycerides are associated with an increased risk of cardiovascular disease (13). Our results demonstrate that patients with homozygous truncating mutations are more likely to have raised triglycerides than patients with other mutation types.

We demonstrated a statistically significant increase in GGT in patients with homozygous truncating mutations and heterozygous truncating and missense mutations compared with patients with homozygous missense mutations. Although used as a marker of chronic liver disease, GGT correlates with cardiovascular diseases and is an independent marker of cardiovascular risk (14) making it a potentially powerful tool in the risk stratification of patients with BBS.

The prevalence of clinical cardiovascular risk factors according to genotype and mutation type do not reveal a clear pattern. This may be because the patients in this study are young (mean age 28.25) and the natural progression of the disease has not yet unfolded, or because the patients with *BBS1* mutations are significantly older than the patients with mutations in *BBS10*. Alternatively, it may reflect a true lack of genotype–phenotype correlation.

Although analysis of renal parameters did not reveal any statistically significant differences, it is noteworthy that the patient on dialysis and both patients who had received renal transplants had two truncating mutations in *BBS10*. This is in keeping with previous studies suggesting that the renal phenotype may be more severe in patients with mutations in *BBS10* (3).

The high prevalence of hypertension (Figure 3a,b) among patients with mutations in *BBS10* and two truncating mutations (42% and 50%) respectively is striking considering only one person in these groups is older than 31. This may represent the early development of a severe cardiovascular phenotype or a statistical error due to sample sizes (19 and 14, respectively).

Conclusion

Cardiovascular disease is a major cause of death and morbidity, and significant resources are allocated to primary prevention in the general population with the aim of reducing the overall disease burden. The same principles should apply to special groups within the population such as patients with BBS where there is an opportunity to practice personalized medicine as the genotype of many patients is already known. This study indicates that patients with missense mutations in *BBS1* may be at lower risk of cardiovascular disease than patients with homozygous truncating mutations and mutations in *BBS10*. In practice most patients with *BBS10* mutations harbour the common homozygous truncating mutation and it is possible that the resulting truncated protein product or a hypomorphic effect of the common missense

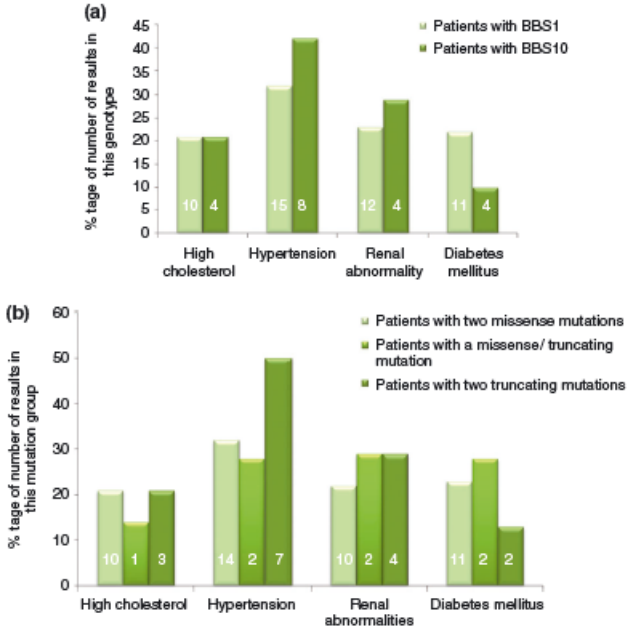

Fig. 3. Prevalence of cardiovascular risk factors by genotype (a) and mutation type (b). The prevalence is illustrated as a percentage to compensate for disproportionate population sizes. Absolute numbers are given in each column.

mutation M390R in *BBS1*, rather than the affected gene, determines the phenotypic effect. Larger studies could clarify this, and longitudinal research will determine the clinical effect these risk factors have on cardiovascular morbidity.

Supporting Information

The following Supporting information is available for this article:

Table S1. Genotype–phenotype correlation: univariable comparison of clinical and laboratory parameters. Patients with *BBS1 vs BBS10*. Statistically significant results are highlighted in bold.

Table S2. Mutation type-phenotype comparison: univariable comparison of clinical and laboratory parameters. Homozygous missense, heterozygous truncating and missense and homozygous truncating mutations. Statistically significant results are highlighted in bold.

Table S3. Genotype–phenotype comparison: multivariable comparison of selected parameters associated with cardiovascular disease. *BBS1 vs BBS10*. Statistically significant results are highlighted in bold.

Table S4. Mutation type-phenotype comparison: multivariable comparison of selected parameters associated with cardiovascular disease. Homozygous missense, heterozygous truncating and missense and homozygous truncating. Statistically significant results are highlighted in bold.

Additional Supporting information may be found in the online version of this article.

Acknowledgements

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