





ORIGINAL ARTICLE

Urine concentrating defect as presenting sign of progressive renal failure in Bardet–Biedl syndrome patients

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ABSTRACT

Background. Urine concentrating defect is a common dysfunction in ciliopathies, even though its underlying mechanism and its prognostic meaning are largely unknown. This study assesses renal function in a cohort of 54 Bardet–Biedl syndrome (BBS) individuals and analyses whether renal hyposthenuria is the result of specific tubule dysfunction and predicts renal disease progression.

Methods. The estimated glomerular filtration rate (eGFR), urine albumin:creatinine ratio (ACR) and maximum urine osmolality (max-Uosm) were measured in all patients. Genetic analysis was conducted in 43 patients. Annual eGFR decline (Δ eGFR) was measured in patients with a median follow-up period of 6.5 years. Urine aquaporin-2 (uAQP2) excretion was measured and the furosemide test was performed in patients and controls.

Results. At baseline, 33 (61.1%), 12 (22.2%) and 9 (16.7%) patients showed an eGFR >90, 60–90 and <60 mL/min/1.73 m², respectively; 27.3% showed an ACR >30 mg/g and 55.8% of patients showed urine concentrating defect in the absence of renal insufficiency. Baseline eGFR, but not max-Uosm, correlated negatively with age. Conversely, truncating mutations affected max-Uosm and showed a trend towards a reduction in eGFR. Max-Uosm correlated with Δ eGFR ($P < 0.005$), suggesting that urine concentrating defect may predict disease progression. uAQP2 excretion and Na⁺ and Cl⁻ fractional

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excretion after furosemide did not differ between hyposthenuric patients and controls, suggesting that specific collecting duct and thick ascending limb dysfunctions are unlikely to play a central role in the pathogenesis of hyposthenuria.

Conclusions. Hyposthenuria is a warning sign predicting poor renal outcome in BBS. The pathophysiology of this defect is most likely beyond defective tubular function.

Keywords: ciliopathy, genetics, GFR, kidney disease, urine osmolality

INTRODUCTION

Bardet–Biedl syndrome (BBS) is a rare autosomal recessive multisystem disorder with an estimated prevalence of 1/125 000–1/175 000 individuals in non-consanguineous European populations [1]. It is characterized primarily by rod–cone dystrophy, renal abnormalities, polydactyly, obesity, learning disabilities and hypogonadism [2, 3]. To date, 24 disease-causing genes have been identified, most of which are involved in assembly and maintenance of the primary cilium [4]. The latter was long considered a mere vestigial remnant, but it is now known that it serves as a mechano-, osmo- and chemosensory unit regulating signal transduction pathways involved in both homeostatic and developmental processes [5]. Defects in the assembly, in the structure and function of this organelle, are associated with a wide range of diseases and syndromic disorders, the ciliopathies, including BBS [6]. Renal dysfunction is a common feature of ciliopathies and it is one of the most common causes of premature death [7]. Structural renal anomalies include foetal lobulations, cysts, clubbing and dysplasia, while functional abnormalities range from urine concentrating defect to end-stage renal disease [8]. Genotype–phenotype correlations are poor, made difficult by genetic heterogeneity and variable expressivity, which is significant both inter- and intrafamiliarily [9–11]. However, a recent retrospective study conducted in a large cohort of BBS patients demonstrated that the severity of genetic mutations rather than the gene involved correlates with the phenotype [12].

Furthermore, information about estimated glomerular filtration rate (eGFR) decline in BBS patients are scanty and no developmental trajectories have been reported to date. This study analysed renal function in a population of 54 Italian BBS patients, with the aim of discovering predictor factors of disease progression and to verify whether renal hyposthenuria, the most common renal dysfunction, is an early sign of progressive renal disease [13]. The results of the current study show that renal dysfunction in BBS patients is highly variable, hyposthenuria is the most common kidney defect and is unlikely the result of specific tubular dysfunction and hyposthenuria predicts kidney disease progression.

MATERIALS AND METHODS

Patients cohort

Patients referred to the units of nephrology and ophthalmology at the University of Campania, Luigi Vanvitelli, with a clinical diagnosis of BBS according to Beals criteria [2] were enrolled. Retinal degeneration was assessed through electroretinogram and visual field tests. Obesity was defined as a body mass index (BMI) <30 kg/m². Learning disabilities defined deficiency in writing, reading, speaking, spelling and/or deficits of memory. Hypogonadism was defined as androgen deficiency or cryptorchidism. All studies were conducted in accordance with international guidelines and complied with the Declaration of

Helsinki. All patients or their legal guardians gave written informed consent for genetic analysis. Ethical approval was obtained from the Institutional Review Board of the Azienda Ospedaliera, Università della Campania L. Vanvitelli (14 February 2014).

Measurement of renal function

At baseline, renal function was assessed in all patients:

1. eGFR was estimated using Chronic Kidney Disease Epidemiology Collaboration equation [14] or the Schwartz equation in children (<15 years). Serum creatinine was measured with an enzymatic method.
2. Albumin:creatinine ratio was measured on random urine collection.
3. Maximal urine concentration ability was assessed on the second void urine sample, collected after overnight fasting and dehydration. Maximum urine osmolality (Uosm) was measured using a freezing point depression osmometre (model 3320, Advanced Instruments, Norwood, MA, USA) as detailed elsewhere [15, 16].

For the longitudinal study, mean annual eGFR decline (Δ eGFR) was estimated as the difference between baseline eGFR and the latest available eGFR divided by the follow-up period, in years. Patients with a follow-up of <4 years were excluded from the study. The rate of eGFR decline was expressed as ml/min/1.73 m²/year.

Genetic analysis: DNA isolation, library preparation and next-generation sequencing

An aliquot of 2 mL of peripheral blood from all patients was taken into ethylenediaminetetraacetic acid (EDTA) tubes. Genomic DNA was isolated using standard procedures. The density (ng/ μ L) and absorbance at 260/280 nm (1.80–2.00) of DNA samples were analysed by a spectrophotometre (Nanodrop ND1000, Thermo Fisher Scientific, Hanover Oark, IL, USA). Double-stranded DNA was quantified using a Qubit fluorimetre (Life Technologies, Carlsbad, CA, USA). For next-generation sequencing analysis, a custom enrichment tool, Nephroplex, covering 115 genes causing inherited kidney diseases (Supplementary data, Table S1) was used. According to the manufacturer's instructions (HaloPlex Target Enrichment System for Illumina Sequencing, August 2012; Agilent Technologies, Santa Clara, CA, USA), 200 ng of genomic DNA was digested to create a library. The fragments were hybridized to specific probes and amplified by PCR. The enriched target DNA in each library sample was validated and quantified using the Bioanalyser High Sensitivity DNA Assay kit (Agilent Technologies) and the 2100 Bioanalyser with 2100 Expert software. For the Pool-seq experiments, 200 ng of the pool was used for the HaloPlex enrichment strategy. The libraries were sequenced using the HiSeq1000 system (Illumina, San Diego, CA,

USA) in a single lane. The generated sequences were analysed using eXSP.

Genetic data interpretation

Genetic variants with a minor allele frequency >0.01 were filtered out using public databases such as the Exome Aggregation Consortium Database [17] and Genome Aggregation Database [18]. Next, in-frame insertions/deletions (indels), synonymous and intronic mutations and mutations in untranslated regions were excluded. Conversely, missense, nonsense and frameshift indels in translating regions were studied. Putative pathogenic mutations were searched in public databases. *In silico* programmes were used to predict the possible impact of the detected mutations on protein function.

Immunoblotting of urine Aquaporin-2 water channel (AQP2)

The second void urine sample was collected after overnight dehydration and fasting. Urine was spun at 5500 rpm. To precipitate proteins, the samples were mixed with methanol, chloroform and distilled water. The sediment was suspended in 150 μ L of buffer [0.3 M sucrose, 25 mM imidazole, 1 mM Ethylenediamine tetraacetic acid (EDTA), 1 mg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Samples were normalized to urinary creatinine for loading, resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen). Filters were blocked in casein and incubated with anti-AQP2 antibody (661AP; kindly provided by Prof. Sebastian Frische). A phosphatase-conjugated anti-rabbit immunoglobulin G was used as a secondary antibody. Bands were visualized with the chemiluminescence kit (Applied Biosystems, Waltham, MA, USA) and quantified by the Versadoc Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

Furosemide test

Five hyposthenuric BBS patients and five age- and gender-matched healthy volunteers were given 50 mg of furosemide orally. Prior to furosemide administration, fasting blood and two urine collections were obtained to measure basal electrolyte levels. Right after, 1 mL/kg of water was administered orally in 45–60 min. Urine was collected every hour for 4 h; osmolality, electrolytes and creatinine concentration were measured in each sample. In order to minimize the risk of fluid depletion, urine output was replaced by water intake during the test. At the end of the test, an additional blood sample was collected for biochemical analysis.

Statistical analysis

Continuous variables were expressed as mean [standard deviation (SD)] and categorical variables were expressed as a percentage.

The correlation of the eGFR and patients' age was assessed by Pearson's correlation coefficient. Trajectory curves were estimated using linear fitting and, in cases where an evident non-linear trend was present, by quadratic fitting.

A stepwise multivariate model was constructed to test the variables associated with CKD, using basal eGFR as the dependent variable and age, albumin:creatinine ratio (ACR), systolic and diastolic blood pressure (SBP and DBP), BMI and maximum Uosm (max-Uosm) as the predictor variables.

To evaluate differences between progressors and non-progressors, the mean annual eGFR decline (Δ eGFR) was converted into a categorical variable using the threshold value of 1.5 mL/min/year to divide the two populations, as modified from former studies of the literature [19].

To evaluate the effect of genetic mutations on the phenotype (eGFR, Δ eGFR and max-Uosm), the Mann-Whitney U-test (non-parametric statistics) was used. Rejection level was set at $P < 0.05$.

RESULTS

Baseline features of the cohort

Fifty-four BBS patients were included in the study, comprising 41 adult and 13 paediatric individuals. The subjects had a mean age of 21.5 years (Table 1). Rod-cone dystrophy was present in all subjects >4 years of age except one [50/51 (98%)], polydactyly was present in 41/54 (75.9%) and a history of childhood obesity was present in all patients. Male hypogonadism was demonstrated in 4/24 (17%) and learning disabilities in 29/48 (60%).

Renal function at baseline: hyposthenuria was the major renal dysfunction and was independent on age

Basal eGFR was distributed as follows: <90 in 61.1% of subjects, 60–90 in 22.2% and <60 mL/min/1.73 m² in 16.7% (Figure 1A). The eGFR correlated significantly with the patients' age (Pearson correlation -0.37 , $P = 0.006$). Both a linear and a quadratic fitting of the data were significant ($P < 0.01$), with the quadratic fitting explaining 19% of the variance of the data, suggesting that age is a risk factor for eGFR decline.

The mean max-Uosm was 581.8 ± 215 mOsm/kg. Basal urine Na⁺/creatinine and urea excretion did not correlate with max-Uosm, excluding that dietary habit could be used to infer the results (data not shown). After excluding patients with an eGFR <60 mL/min/1.73 m², which were expected to show hyposthenuria due to generalized renal fibrosis, the frequency of hyposthenuria was still high [24/43 patients (55.8%)]. In contrast, fasting urine osmolality was not significantly correlated with age (Pearson coefficient -0.11 , $P = 0.44$) (Figure 1B). Only 9/33 (27.3%) patients had an uACR >30 mg/g (Supplementary data, Figure S1).

We applied a stepwise multivariate model using eGFR as the dependent variable and age, ACR, SBP, DBP, BMI and max-Uosm as independent variables (Table 2). Interestingly, only max-Uosm and age were significantly correlated with baseline eGFR. Major renal findings of the patients' cohort are described in Supplementary data, Table S2.

Table 1. General features of the patient cohort

Features	Value
No. of patients	54
Age (years), mean (range)	21.5 (0–41)
Sex (female/male), n/n	28/26
Rod-cone dystrophy (>4 years), n/N	50/51
Polydactyly, n/N	41/54
Obesity (adults), n/N	27/41
History of childhood obesity, n/N	53/54
Learning disabilities, n/N	29/48
Hypogonadism in males, n/N	4/24

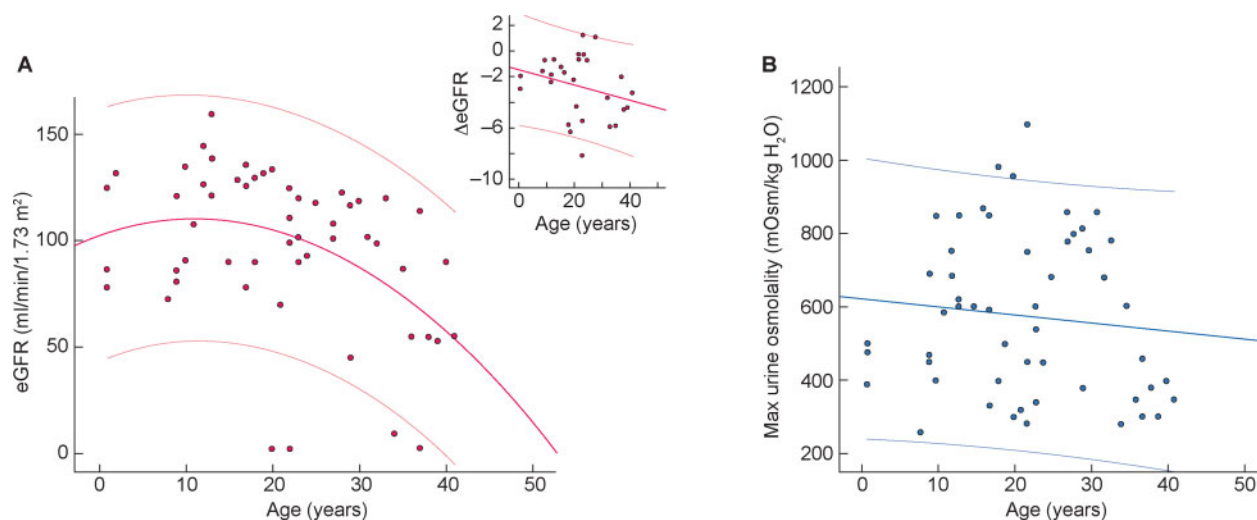


FIGURE 1: Kidney function trajectory in BBS subjects. (A) eGFR declines with age in BBS. Inset: the rate of decrease does not significantly change with age [Pearson's coefficient of eGFR variation (mL/min/year) versus age = -0.27 , $P = 0.17$]. (B) Urine osmolality changes do not follow the same trajectory of the eGFR decline as a function of age. Fitting curves are quadratic for panel A and linear for the inset and for panel B. 95% CIs are also reported.

Table 2. Multiple correlation analysis of baseline eGFR using max-Uosm and age as predictors

Variable	R ²	Standardized coefficients (β)	P-value
Max-Uosm	0.571	0.654	0.000
Age		-0.263	0.022

ACR, SBP and DBP and BMI were excluded from the multiple correlation analysis using a stepwise regression method.

Renal disease progression in BBS patients was independent of age and was faster in hyposthenuric subjects

The mean eGFR slope was analysed in a subpopulation of 24 BBS patients undergoing a follow-up period for a median of 6.5 years. The mean annual Δ eGFR was -2.88 ± 2.3 mL/min/year. Patients with a faster GFR decline (Δ eGFR < -1.5 mL/min/year) did not differ from those with relatively stable eGFR regarding baseline age, GFR, BMI, SBP and DBP. However, patients with a faster eGFR decline had a significantly lower max-Uosm at baseline compared with non-progressors (518.4 ± 164 versus 724.6 ± 216.7 ; $P < 0.005$) (Table 3). The rate of eGFR decline remained approximately constant over the years (Pearson correlation -0.27 , $P = 0.17$), suggesting that it was not accelerating over time (Figure 1C).

Genetic analysis and genotype–phenotype correlation: truncating mutations affected max-Uosm

Forty-three patients underwent genetic analysis. Twenty-seven patients were studied in the current study, while 12 were analysed previously as described elsewhere [10] and 4 showed at the baseline visit a report of a genetic analysis previously conducted in another genetic unit. A biallelic mutation was identified in 31 patients, while 12 were found to have either a heterozygous mutation or a negative genetic analysis. The most affected genes were *BBS10* and *BBS12*, which were mutated in eight and seven patients, respectively; *BBS1* mutations were identified in five patients, *BBS4* and *BBS9* in four patients each and *BBS2* showed biallelic mutations in three patients. Twenty-

five patients showed biallelic truncating or splicing mutations or mixed (truncating/any) mutations and nine patients showed biallelic missense mutations. Supplementary data, Table S2 shows the results of the genetic analyses. Truncating mutations are known to cause a more severe phenotype than missense mutations [12]. Interestingly, we observed that truncating mutations were significantly associated to max-Uosm (Table 4).

Urine concentrating defect co-existed with normal-high urine aquaporin 2 at baseline and normal NaCl absorption along the thick ascending limb (TAL)

The renal origin of hyposthenuria in BBS patients has been demonstrated by independent studies [15, 20]. For a deeper analysis, we investigated the putative role of two crucial nephron segments in urine concentration: the collecting duct (CD) and the TAL of the loop of Henle.

To this end, urinary excretion of AQP2 (u-AQP2), known to correlate with AQP2 abundance on the apical membrane of the CD [21], was measured by immunoblotting. Figure 2 shows that u-AQP2 abundance was not different among the two groups.

Na⁺ and Cl⁻ fractional excretion (FeNa% and FeCl%) were measured at baseline and after furosemide administration in hyposthenuric BBS patients and controls. FeNa% and FeCl% significantly increased compared with baseline in both groups, indicating normal salt absorption along the TAL (Figure 3).

DISCUSSION

Kidney disease is a common morbidity in BBS patients; however, to date, few studies have analysed renal disease progression by evaluating risk and predictor factors of poor renal outcome. Renal structural abnormalities are quite common and renal dysfunction shows a wide range of severity, from urine concentrating defect to end-stage renal disease [12]. Little information is available on the utility of genetic and/or biochemical parameters in discriminating patients at risk of kidney disease progression.

In this study, renal function was characterized in 54 BBS patients at baseline: eGFR, urine ACR and max-Uosm were measured. Genetic analysis was performed in 43 patients and a

Table 3. 24 BBS patients with a median follow-up period of 6.5 years were divided by the base of the mean annual decline of the eGFR

Variables	Patients with mean Δ eGFR < -1.5 mL/min/year	Patients with mean Δ eGFR > -1.5 mL/min/year	P-value
Total number	19	9	-
Age (years)	22.68 ± 12.5	20.3 ± 5.9	0.5
Baseline eGFR (mL/min/1.73 m ²)	98.9 ± 35.3	113.5 ± 15.1	0.22
ACR (mg/g)	282.3 ± 613	7.7 ± 6.6	0.17
BMI (kg/m ²)	33.17 ± 6.3	28.7 ± 5.2	0.14
U-osm (mOsm/L)	506.3 ± 171	737.8 ± 216.7	<0.005
SBP (mmHg)	121 ± 18	112 ± 18	0.3
DBP (mmHg)	80 ± 9.2	78.6 ± 13	0.8

Values presented as mean ± SD unless stated otherwise. Patients with more severe reductions did not differ for any basal parameter of the table but max-Uosm.

Table 4. Mann-Whitney test shows that biallelic truncating and mixed mutation (truncating plus any type of mutations) significantly correlate with max-Uosm

Variable	Truncating/mixed mutation	Missense mutation	P-value (Mann-Whitney)
eGFR (ml/min/1.73 m ²), mean ± SD	85.3 ± 41 (n = 21)	132.1 ± 3(n = 6)	0.1
Max-Uosm (mOsm/kg), mean ± SD	490.9 ± 158 (n = 18)	654 ± 181 (n = 6)	0.05

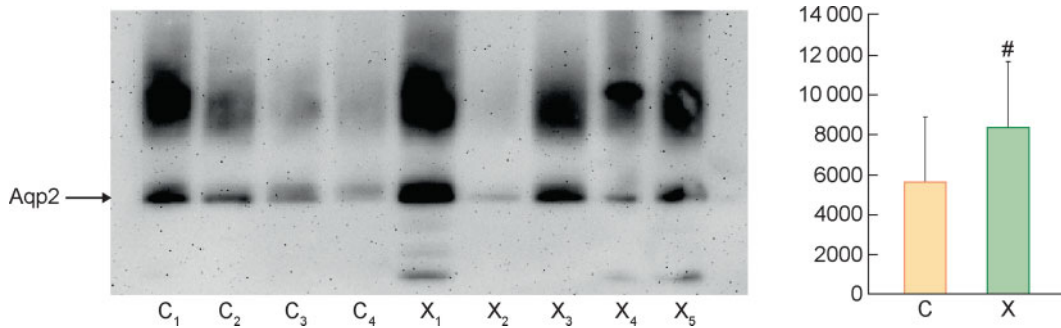


FIGURE 2: uAQP2:creatinine ratio in four healthy volunteers and five hyposthenuric BBS patients. Each lane is representative of uAQP2 abundance per 0.5 mg creatinine loaded. Densitometric analysis is shown in the right panel.

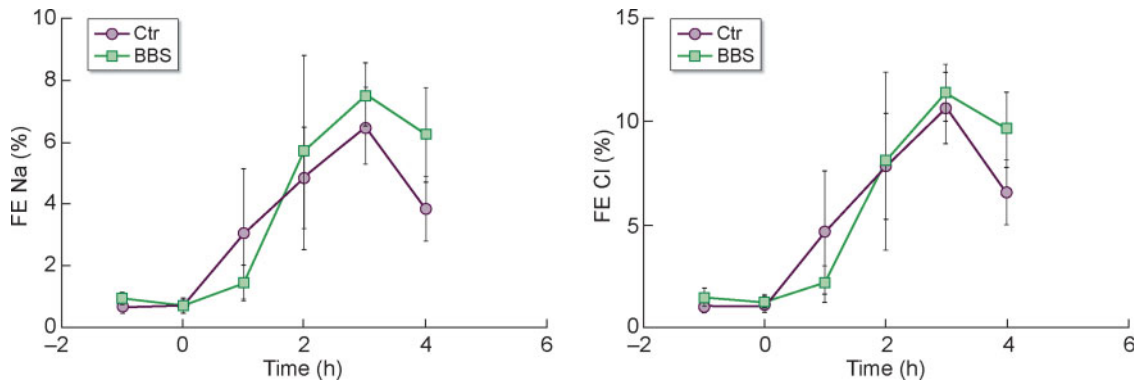


FIGURE 3: Furosemide test showing mean FENa% and FECl% at baseline and every hour up to 4 h in five hyposthenuric BBS patients and five controls. Both BBS patients and controls showed a significant increase after furosemide administration, with no difference among the two groups.

biallelic mutation was demonstrated in 31 patients. A subcohort of patients with a median follow-up period of 6.5 years was selected to analyse renal disease progression.

On cross-sectional analysis, our results showed that the eGFR was highly variable among patients, with 38.9% of patients having an eGFR < 90 mL/min/1.73 m². The eGFR significantly

correlated with the patient's age, indicating that renal disease progresses over years. Thus, as in the general population, age is a contributing factor of renal disease progression in BBS [13]. Conversely, the defect of urine concentration was equally present in adult and paediatric individuals. It is possible that it is determined at birth, with a pathogenesis that is to date largely unknown. We have applied a non-parametric test to verify the correlation between the severity of genetic mutations and renal phenotype. Given the high genetic heterogeneity of the disease in our cohort, we were unable to address whether specific genetic loci correlated with the phenotype. Truncating mutations, which are known to determine no protein synthesis or proteins with lower molecular weight, are expected to alter protein function and are often associated with a more severe phenotype than missense mutations, also in BBS [12]. Thus we evaluated whether truncating mutations correlated with max-Uosm, baseline eGFR and Δ eGFR. Interestingly, these mutations showed a significant correlation only with max-Uosm. Why truncating mutations did not significantly correlate with eGFR is unclear. One explanation is that the number of patients was insufficient to get conclusive results and the trend we observe could reach statistical significance in a larger cohort. Another explanation is that the decline of the eGFR is only dependent in part on genetic mutations and possibly additional factors influence disease progression [22–24].

Several findings of this study focused attention on hyposthenuria as a warning sign of renal disease progression: it correlated with baseline eGFR, with truncating mutations and with Δ eGFR. This evidence prompted us to elucidate the pathophysiology of this dysfunction.

Hyposthenuria is a typical sign of tubular–interstitial dysfunction that may have an intrinsic or an extrarenal pathogenesis. Renal diseases characterized by hyposthenuria include a number of disorders: genetic diseases affecting water and salt reabsorption, such as TAL (Bartter syndrome) and CD (insipidus diabetes) dysfunctions, and congenital or acquired conditions affecting the integrity of the tubule–interstitium, including CKD by any cause [25].

The mechanism underlying urine concentrating defect in BBS is largely unclear [26–28]. Our previous studies demonstrated that it has a renal origin and is desmopressin (ddAVP) resistant. However, V2 receptor binding to ddAVP was unaffected in endothelial cells, arguing whether along the nephron there is no defective ddAVP/ADH-V2R binding. Conversely, the increased urinary AQP2 excretion induced by ddAVP was blunted in BBS patients, suggesting a possible role of BBS proteins in AVP-dependent AQP2 trafficking downstream of V2R-ADH binding, at least after short-term exposure to ddAVP [15]. This study suggests that u-AQP2 excretion after long-term water restriction did not differ between patients and controls. Moreover, the evidence that u-AQP2 excretion has an increasing trend suggests possible compensatory ADH overproduction. Unfortunately, our patients did not undergo measurement of copeptin plasma levels to further demonstrate renal insensitivity to vasopressin and its compensatory overproduction. However, the central role of kidney dysfunction is further supported by our previous studies showing an increase of Na-retentive hormones in BBS individuals [15]; in addition, copeptin plasma levels were higher in hyposthenuric patients affected by other ciliopathies, such as nephronophthisis: that a common denominator causes hyposthenuria in recessive syndromic ciliopathies is a possibility [29]. Even though these findings support the hypothesis of a pivotal role of kidney resistance to dDAVP/AVP in the pathogenesis of hyposthenuria,

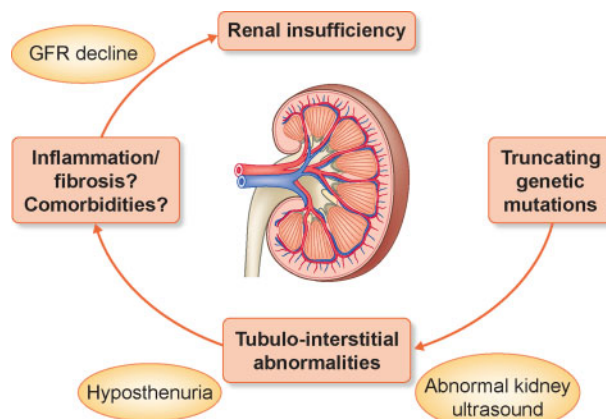


FIGURE 4: Schematic representation of a proposed mechanism leading to renal disease in BBS.

the exact mechanisms remain elusive. Our data on AQP2 excretion suggest a marginal role of AQP2 mistrafficking; however, given the variability of u-AQP2 excretion in both patients and controls, this study cannot provide firm conclusions on this issue. Besides the CD, in this study the role of the TAL, another crucial nephron segment in urine concentration, has been tested through the furosemide test [30]. In the case of reduced NKCC2 function as a cause of reduced corticomedullary osmotic gradient and consequently urine hypo-osmolality, we would expect a blunted increased FENa and FECl% after furosemide compared with baseline. Surprisingly, we observed a normal increase in FeNa and Cl% in BBS patients, resembling controls and indicating normal NKCC2 function. Whether a perturbation of the medulla architecture with primary or secondary tubulo-interstitial fibrosis is the underlying mechanism remains a possibility requiring further investigations. Histological data from BBS patients showed the presence of cortical and medullary cysts, interstitial fibrosis and chronic inflammation, supporting our hypothesis [31].

We have shown previously in different mice models that the alteration of interstitial architecture induced by renal ablation of beta-1 or dicer severely affect urine concentration ability [32–34]. The same defect occurs also after treatment with lithium salt [35] before a frank polyuria develops; whether hyposthenuria is an anticipatory factor of lithium-induced CKD, as for BBS patients, is an interesting perspective to investigate. As an additional piece of the puzzle, our previous data indicated that the eGFR decline in BBS patients correlated with the urinary abundance of markers of fibrosis, cell adhesion, extracellular matrix organization proteins and inflammation proteins [16]. Figure 4 proposes a hypothetical mechanism explaining hyposthenuria and progression towards CKD in BBS patients harbouring truncating mutations.

In conclusion, this study demonstrates that the majority of BBS patients undergo renal disease progression over years and that hyposthenuria predicts kidney disease progression, discriminating patients requiring intensified monitoring. The exact pathomechanism underlying hyposthenuria remains elusive.

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CONFLICT OF INTEREST STATEMENT

None declared.

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