Metabolomic fingerprinting of renal disease progression in Bardet-Biedl syndrome reveals mitochondrial dysfunction in kidney tubular cells

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Highlights
Targeted Metabolomics reveals a unique serum fingerprinting of patients with BBS with CKD

Acylcarnitines are among the most significant alterations

In renal epithelial cells, Bbs10 depletion leads to mitochondrial abnormalities

Human BBS 10 interacts with six mitochondria-related proteins

Marchese et al., iScience 25, 105230
November 18, 2022 © 2022
The Author(s).
https://doi.org/10.1016/j.isci.2022.105230
Metabolomic fingerprinting of renal disease progression in Bardet-Biedl syndrome reveals mitochondrial dysfunction in kidney tubular cells

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SUMMARY
Chronic kidney disease (CKD) is a major clinical sign of patients with Bardet-Biedl syndrome (BBS), especially in those carrying BBS10 mutations. Twenty-nine patients with BBS and 30 controls underwent a serum-targeted metabolomic analysis. In vitro studies were conducted in two kidney-derived epithelial cell lines, where Bbs10 was stably deleted (IMCD3-Bbs10−/− cells) and over-expressed. The CKD status affected plasmatic metabolite fingerprinting in both patients with BBS and controls. Specific phosphatidylcholine and acylcarnitines discriminated eGFR decline only in patients with BBS. IMCD3-Bbs10−/− cells displayed intracellular lipid accumulation, reduced mitochondrial potential membrane and citrate synthase staining. Mass-Spectrometry-based analysis revealed that human BBS10 interacted with six mitochondrial proteins, in vitro. In conclusion, renal dysfunction correlated with abnormal phosphatidylcholine and acylcarnitines plasma levels in patients with BBS, in vitro. Bbs10 depletion caused mitochondrial defects while human BBS10 interacted with several mitochondria-related proteins, suggesting an unexplored role of this protein.

INTRODUCTION
Bardet-Biedl syndrome (BBS) is a rare inherited disorder characterized by multi-organ dysfunction. The phenotype of patients with BBS is variable: the most common clinical findings include retinal dystrophy, obesity, polydactyly, intellectual disabilities, and renal abnormality. The latter is characterized by a wide range of kidney and urinary tract malformations, including fetal lobulations, pelvic dilation, renal cysts, renal hypoplasia, and vesical-ureteral reflux (Zacchia et al., 2016; Zaghloul and Katsanis, 2009). These structural anomalies are accompanied by functional defects, with a variable level of severity in available clinical studies, ranging from urine concentrating defects to end-stage renal disease (ESRD). The variability of kidney phenotype is at least in part dependent on the genetic heterogeneity of BBS; in fact, to date 24 genes have been defined as the cause of human BBS and among these, BBS10 is known to correlate with severe renal phenotype. The function of BBS proteins is only partially known; given their primary subcellular localization to the basal of the primary cilium (PC), the disorder is considered a ciliopathy.

There is significant evidence that energy metabolism is impaired in both genetic and acquired kidney disorders (Zacchia et al., 2020, 2021b). Moreover, metabolomics has emerged as a valid tool to analyze metabolites within biological fluids, aiming at detecting disease-related biomarkers as well as molecular aberrations underlying pathologic conditions.

In this study, we characterize the serum metabolome profiling linked to renal disease in 29 patients with BBS compared to a cohort of 30 controls, matched for age, gender, body mass index (BMI), and estimated glomerular filtration rate (eGFR). The control group included healthy volunteers and patients with chronic kidney disease (CKD) suffering from glomerulonephritis and non-syndromic congenital anomalies of the renal and urinary tract (CAKUT). Our study revealed a clear separation of plasma metabolite profile of patients with BBS with and without eGFR decline; moreover, a discrete group of metabolites appeared linked to the eGFR decline in patients with BBS, indicating a BBS-specific effect.
The resulting BBS-specific metabolites linked to kidney damage included high molecular weight lipids of the classes of acylcarnitines (ACs) and phosphatidylcholine (PCs). ACs are intracellular molecules and PCs are cell membrane components and crucial signaling molecules. We tested if their precursors, long chain free fatty acids, were altered in tubular cells lacking the Bbs10 gene. The deep investigation of lipid homeostasis alteration reveals that the reduction of Bbs10 affects metabolic pathways, including mitochondrial dysfunction.

Finally, we showed that the BBS10 protein interacts with six mitochondrial proteins, further suggesting its putative mitochondrial role and indicating a possible mechanistic explanation of kidney dysfunction in BBS.

RESULTS

Patients

Twenty-nine adult patients with the clinical diagnosis of BBS according to Beales criteria (Beales et al., 1999) and 30 controls were enrolled. Features of patients and controls are listed in Table 1. Briefly, patients and controls included individuals with no differences in age, gender, max-Uosm, eGFR, and BMI. To address whether changes in the eGFR affected plasma metabolites pattern, both patients with BBS and controls were divided into two groups based on the eGFR. Specifically, patients with BBS having an eGFR higher than 90 mL/min/1.73 m² were referred to as BBS with no chronic kidney disease (BBS_noCKD) while patients with BBS having an eGFR lower than 90 mL/min/1.73 m² were referred to as BBS_CKD. Control subjects included healthy volunteers (ctr_hv) and subjects affected by CKD due to other causes (ctr_CKD), mainly glomerulonephritis and congenital anomalies of kidney and of urinary tract malformations (CAKUT). Genetic analysis (Table 2) of enrolled patients revealed that 9 patients showed mutations in chaperonin-like BBS proteins, with 6 and 3 patients bearing mutations in BBS10 and BBS12, respectively; 3, 4, and 5 patients showed biallelic mutations in BBS1, BBS4, and BBS9, respectively; one patient had two in trans BBS2 mutations. Seven patients had no genetic diagnosis after molecular screening.

Metabolomic fingerprinting of patients with chronic kidney disease differed from non-chronic kidney disease individuals, in both patients with Bardet-Biedl syndrome and controls

A targeted metabolomic analysis was performed using serum samples from patients and controls, in order to quantify 180 metabolites (Tables S1 and S2). To identify the metabolites discriminating the CKD status (eGFR>90 vs eGFR<90 mL/min/1.73 m²) specifically in patients with BBS we devised the following strategy: SPLS-DA, a supervised analysis of metabolome data, was used to discriminate the CKD state separately in the BBS and in non-BBS subjects. The results are presented in Figure 1. The PLS-DA algorithm optimally discriminated the CKD status based on the metabolome data in both BBS and control populations (Figures 1A and 1B). ROC curves confirmed the high performance and robustness of the PLS-DA model (controls, AUC = 0.99; patients with BBS, AUC = 0.94; Figures 1C and 1D). The relevance of each metabolite for the discrimination of the CKD status was identified by the Variable Importance in Projection (VIP) score (Figures 1E and 1F). SPLS-DA allows for relevant feature selection and graphical displays for metabolomic data. The SPLS-DA output performance was tested using the receiver operating characteristic (ROC) curves. Subsequently, we computed the influence on the CKD categorization of every metabolite, retrieving the VIP.

VIP coefficients represent the importance of each metabolite to predict the CKD state. Metabolites with a VIP score greater than 1 were then used for subsequent analysis. Therefore, two lists of metabolites with VIP
| Patient ID | Gene   | Chr (hg.19) | Genetic mutation     | Protein variation     | Zygosity |
|------------|--------|-------------|----------------------|-----------------------|----------|
| P1         | BBS1   | Chr 11: 66,287,160 | c.664 G>C           | p.G222R               | HOM      |
| P2         | BBS10  | Chr 12: 76,742,038 | c.101 G>C           | p.R34P                | HOM      |
| P3         | BBS10  | Chr 12: 76,742,038 | c.101 G>C           | p.R34P                | HOM      |
| P4         | No mutations in BBS genes |            |                      |                       |          |
| P5         | BBS12  | Chr 4: 123,665,070 | c.C2023T/c.C2023T   | p.R675X/p.R675X       | HOM      |
| P6         | BBS10  | Chr 12: 76,740,674 | c.1091del           | p.N364Tfs*5           | HOM      |
| P7         | No mutations in BBS genes |            |                      |                       |          |
| P8         | BBS10  | Chr 12: 76,742,038 | c.101 G>C           | p.R34P                | HOM      |
| P9         | No mutations in BBS genes |            |                      |                       |          |
| P10        | No mutations in BBS genes |            |                      |                       |          |
| P11        | BBS10  | Chr 12: 76,741,124 | c.641T>A            | p.V214E               | HOM      |
| P12        | BBS4   | Chr 15: 73,007,751; Chr 15: 73,009,123 | c.332 + 8T>G c.338delT | spl | p.L113fs | DOUBLE HET |
| P13        | BBS4   | Chr 15: 73,007,744 | c.332 + 1G>GTT      | spl                   | HOM      |
| P14        | BBS4   | Chr 15: 73,007,744 | c.332 + 1G>GTT      | spl                   | HOM      |
| P15        | BBS2   | Chr 16: 56,530,878 | c.1909_1910del     | p.M637Fs*12           | HOM      |
| P16        | BBS1   | Chr 11: 66,287,160 | c.664G>C            | p.G222R               | HOM      |
| P17        | BBS9   | Chr 7: 33,296,990; Chr 7: 33,545,112 | c.585_586del/c.2033delG | p.V196LFs*10/G678As*10 | DOUBLE HET |
| P18        | BBS1   | Chr 11: 66,293,652; Chr 11: 66,299,160 | c.1169T>G/c.1642delC | p.M390R/p.L548Wfs*31 | DOUBLE HET |
| P19        | BBS10  | Chr 12: 76,741,436; Chr 12: 76,741,994 | c.325_328del/c.145C>T | p.M109Vfs*25 | DOUBLE HET |
| P20        | No mutations in BBS genes |            |                      |                       |          |
| P21        | No mutations in BBS genes |            |                      |                       |          |
| P22        | BBS9   | Chr 7: 33,185,869; Chr 7: 33,384,190 | c.6_6delT/c.1276-1277delAGCA | p.L3Yfs*38/p.G226fs*5 | DOUBLE HET |
| P23        | BBS12  | Chr 4: 123,665,070 | c.2023C>T           | p.R675X               | HOM      |
| P24        | BBS12  | Chr 4: 123,665,070 | c.2023C>T           | p.R675X               | HOM      |
| P25        | BBS4   | Chr 15: 73,007,744; Chr 15: 73,027,508 | c.332 + 1G>GTT     | spl                   | DOUBLE HET |
| P26        | No mutations in BBS genes |            |                      |                       |          |
| P27        | BBS9   | Chr 7: 33,312,706 | c.T785C             | p.V262A               | HOM      |
| P28        | BBS9   | Chr 7: 33,296,989; Chr 7: 33,185,912 | c.586_587del/c.51_54del | p.Val196Leufs*10/p.Glu18Asnfs*22 | DOUBLE HET |
| P29        | BBS9   | Chr 7: 33,296,989; Chr 7: 33,185,912 | c.586_587del/c.51_54del | p.Val196Leufs*10/p.Glu18Asnfs*22 | DOUBLE HET |

"No mutations in BBS genes" indicates that the BBS phenotype was due to alterations in unknown genes not belonging to the BBS gene family.
Figure 1. Partial least squares-discriminant analyses (PLS-DA) and ROC curve of serum metabolome showed the discrimination of the CKD status in patients with BBS (n = 29) and controls (n = 30)

(A and B) plot of the first two PLS-DA components discriminating subjects according to CKD status in the control group (A: controls, B: BBS).

(C and D) Receiver operating characteristic (ROC) curves are presented to evaluate the performances and robustness of the PLS-DA model. The AUC was high in both control (C, AUC = 0.99) and patients with BBS (D, AUC = 0.94).
score >1 were obtained, one for patients with BBS and one for patients with non-BBS. The two lists were
then compared and only the metabolites present exclusively in patients with BBS were then evaluated.

Variables with VIP score >1 are shown in Figure 2A using a Venn diagram to display discriminating metab-
olites shared and unshared by the two populations (BBS controls). The list includes 29 metabolites shared
among the two groups (25%). Shared molecules included biogenic amines, short-chain acyl-carnitines, and
long-chain phosphatidylcholine.

We further filtered the list of BBS unshared metabolites using the following criteria: (i) metabolites not
included in the list of controls VIP metabolites, (ii) presence of a significant correlation with the eGFR,
and (iii) presence of a significant difference between BBS and non-BBS regarding the calculated Pearson
coefficient. The scatterplot of the eGFR vs the resulting 10 metabolites is presented in Figure 2B and Table 2.

We have further verified the effect of BBS and the connection with eGFR for these metabolites using a linear
mixed model with the eGFR as a dependent variable and (1) the metabolite concentration and (2) the BBS
status (BBS vs controls) as predictors. All of the selected 10 metabolites show significant interaction metab-
olite concentration per BBS genotype (Table 3).

The resulting BBS-specific metabolites linked to kidney damage are high molecular weight lipids of the
classes of acylcarnitines (ACs) and phosphatidylcholine (PCs).

Generation of inner medulla collecting duct (IMCD3)-Bbs10−/− model

The ACs are intracellular molecules and PCs are cell membrane components. We tested whether lipid
metabolism is altered in tubular cells lacking Bbs10.

Figures 3A and 3B show the efficiency of Bbs10 invalidation, in IMCD3 cells. Real-time analysis of Bbs10
gene expression level in four independent clones of Bbs10−/− cells and wild-type cells showed a dramatic
decrease in Bbs10 mRNA in mIMCD3 Bbs10−/− cells if compared with controls. The Figures 3C and 3D sum-
marized the effect of Bbs10 invalidation on the primary cilium (PC) formation: a dramatic reduction of cili-
atized cells was detected in IMCD3-Bbs10−/− cells compared with the wild type. Moreover, Bbs10−/− cells
showed a significantly increased mitotic rate compared with wild-type cells (Figure 3E).

IMCD3-Bbs10−/− cells showed increased cytosolic lipid droplets and signs of mitochondrial
dysfunction

IMCD3-Bbs10−/− cells and controls were stained with oil red, after exposure for 24 h to a high FFA content
medium. After treatment, IMCD3-Bbs10−/− cells showed an increased cytoplasmic accumulation of lipid
droplets, suggesting an imbalance between lipid uptake and metabolism (Figures 4A–4C). We, therefore,
selected a series of enzymes to test mitochondrial dysfunctions in IMCD3-Bbs10−/− cells (Figure S1). Rela-
tive mRNA expression of CPT1, CPT2, ACOX1, PPAR alpha, PGC1a, and PPAR gamma(H) was evaluated in
experimental and wild-type cells. The results didn't allow us to speculate about the observed alterations
due to the low endogenous expression levels at basal in IMCD3 cells. Considering the role of mitochondria
in the FFA metabolism, we studied overall mitochondria function in our cells. Immunofluorescence analysis
revealed a significant reduction of mitochondrial membrane potential (ΔΨM) in IMCD3-Bbs10−/− cells
compared to wild-type cells, as shown by the mitotracker CMXros fluorescence intensity (Figures 5A and
5B). Interestingly, experimental cells showed also a decreased citrate synthase (CS) staining (Figure 5B),
a marker of intact mitochondria and total mitochondrial mass. In addition, the protein amount of specific
mitochondrial markers was tested (Figure 5). Finally, VDAC1, a marker of outer membrane mitochondria,
resulted significantly increased in IMCD3-Bbs10−/− cells compared to wild-type cells (Figure S1B). To
further validate the cellular model, we have analyzed the presence of cellular inclusions using Red-oil stain-
ing in the urine sediment of patients with BBS10 mutation. Results, shown in Figure S2 support the presence
of lipids droplets in tubular cells of the urine sediment of patients with BBS10 (see Figure S2 for details).
Protein-protein interaction analysis revealed that BBS10 interactors included six mitochondrial proteins.

In order to analyze the possible link between mitochondrial dysfunction and Bbs10 depletion in cells, protein-protein interaction (PPI) studies were carried out, aiming to identify proteins that might interact with human BBS10. A cell line stably expressing BBS10-flag protein (HEK293T_BBS10) was generated. BBS10-flag protein was employed as baits in the immunoprecipitation experiment (IP) in order to isolate and then identify its protein interactors. A cell line stably expressing GFP-flag protein was used as a control. Interactome analyses identified BBS10 putative protein-ligands, by nano-LC-MS/MS and subsequent quantitative proteomic analysis (Table S3). Interestingly, six mitochondrial proteins were found among BBS10 interactors (Table 4), including proteins involved in mitochondria biogenesis and function.

Figure 2. Determination of BBS-specific metabolites associated with eGFR decline

(A) Venn diagram of metabolites discriminating the CKD status in the BBS (n = 29) and control (n = 30) populations based on a VIP score > 1. The intersection between the two sets is also reported.

(B) BBS-specific metabolites were further filtered as follows: (i) not included in the list of control VIP metabolites (ii) significant correlation with eGFR (iii) and significant difference between BBS and controls regarding the calculated Pearson coefficient. The scatterplot of the eGFR vs the resulting 10 metabolites is presented.
To estimate the BBS10-flag abundance compared to the endogenous protein, the amount of protein was measured in total extracts from HEK293T_BBS10 and wt HEK293T (HEK293T_CTRL) cell lines by mass-spectrometry-based quantitative methods. The protein abundance was estimated by using normalized MS counts, as the ratio between the MS counts of the interest proteins and the total MS counts in the analyzed sample. According to normalized MS counts, the BBS10-flag abundance resulted in 30-fold higher than endogenous protein (Figure S3 and Table S4), while the levels of ACTB and TUBA proteins between the two samples were comparable.

**Nuclear and mitochondrial localization of BBS10**

As PPI analysis identified six mitochondrial BBS10-interacting proteins (Table 4), we investigated if BBS10-flag protein localized in the mitochondria. To this aim, subcellular fractionation of cells stably expressing BBS10-flag protein was performed and a mitochondrial extract (MitoEx) was analyzed by Western blot (WB) and compared with a whole cell extract (WCE) (Figure S4A). This analysis revealed that BBS10-flag might be present in the mitochondria, in a quite low abundance when compared to the WCE lane. The loaded amount of MitoEx and WCE corresponds to about 13.7 and 6.4% of protein content from 10 million cells, respectively. The citrate synthase (CS) signal was used as a marker of mitochondrial fraction enrichment, while the complete absence of α-tubulin (TUBA) signal in the MitoEx lane demonstrates the complete absence of contamination from cytosolic fraction.

Among the BBS10 interactors, several nuclear proteins were identified. On the other hand, BBS10 protein is predicted to have a nuclear import motif and other BBS proteins are known to have also nuclear localization (Marchese et al., 2020). Immunoblotting studies of cytosolic (CytoEx) and nuclear (NuclEx) extracts revealed that BBS10-flag was also contained in the nuclei (Figure S4B). The histone H2 (H2AX) signal was used as a marker of nuclear fraction enrichment while a very faint band of α-tubulin (TUBA) in the NuclEx lane might indicate a very slight cytosolic contamination of the nuclei.

To further verify the localization of BBS10 protein in human renal tissue, three biopsies from patients who underwent biopsy for proteinuria, but did not show significant modifications in the kidney structure, were studied. BBS10 protein was expressed in tubular cells at the level of the cytoplasm with minimal expression at the nuclear level. Within the cytoplasm, BBS10 has a diffuse staining pattern, with more intense spots that colocalized with MUT protein, a marker of mitochondria (Figure S5).

**DISCUSSION**

The main result of the present study is the identification of a set of plasma metabolites linked to the presence of CKD selectively in patients with BBS, along with evidence of a role of BBS10, one of the major BBS

| Metabolite | BBS | Controls | BBS vs Pearson controls (p) | VIP | BBS effect (p) | eGFR effect (p) | BBS x metabolite interaction effect (p) |
|------------|-----|----------|-----------------------------|-----|----------------|-----------------|---------------------------------------|
| C10:1      | −0.69 (<0.01) | −0.30 (>0.05) | 0.05 | 2.04 | 0.45 | 0.97 | 0.00 | 0.04 |
| C14:2-OH   | −0.55 (<0.01) | 0.00 (>0.05) | 0.02 | 1.61 | 0.74 | 0.69 | 0.02 | 0.03 |
| C16:1      | −0.70 (<0.01) | −0.25 (>0.05) | 0.02 | 2.16 | 0.23 | 0.86 | 0.00 | 0.02 |
| lysoPC a C26:0 | 0.63 (<0.01) | 0.10 (>0.05) | 0.02 | 2.05 | 0.09 | 0.00 | 0.00 | 0.03 |
| PC aa C24:0 | 0.52 (<0.01) | −0.25 (>0.05) | 0.00 | 1.54 | 0.14 | 0.93 | 1.00 | 0.02 |
| PC aa C26:0 | 0.55 (<0.01) | 0.02 (>0.05) | 0.03 | 2.09 | 0.11 | 0.00 | 0.02 | 0.05 |
| PC aa C32:0 | −0.58 (<0.01) | −0.08 (>0.05) | 0.03 | 1.47 | 0.56 | 0.23 | 0.01 | 0.08 |
| PC aa C32:3 | −0.61 (<0.01) | 0.08 (>0.05) | 0.00 | 1.33 | 0.65 | 0.32 | 0.06 | 0.02 |
| PC aa C42:5 | −0.58 (<0.01) | −0.05 (>0.05) | 0.02 | 1.36 | 0.06 | 0.46 | 0.01 | 0.06 |
| PC ae C42:1 | −0.46 (<0.01) | 0.11 (>0.05) | 0.03 | 1.40 | 1.05 | 0.58 | 0.15 | 0.04 |

VIP, variable importance in the projection; Lm, linear model formula.
Figure 3. Generation of stable renal tubular epithelial cell line lacking Bbs10 gene (IMCD3-Bbs10⁻/⁻⁻)

(A–E) microscopy images of CRISPR/Cas9-modified cells. After transfection, cells were observed with a 20X objective, and images were acquired with the Leica LAS AF software. mIMCD3 Bbs10⁻/⁻ clone: Cell populations isolated from single progenitor cells within the Bbs10⁻/⁻ pool. RFP: Fluorescence signal from the red fluorescent protein detected with a Leica N3 filter cube. To limit clone-dependent effects, four different KO clone cells were used, in addition to the negative control line transfected with Cas Nuclease Only (B) real-time analysis of Bbs10 gene expression level in Bbs10⁻/⁻ cells and wild-type cells. Relative mRNA expressions of Bbs10 in mIMCD3 Bbs10⁻/⁻ cells (n = 20) and wild-type cells (n = 5). Data are presented as mean ± SEM, are normalized to wild-type controls as y axis is indicated by % fold. The statistical significance of the difference in mRNA expressions between conditions (mIMCD3 Bbs10⁻/⁻ cells and wild-type controls) was evaluated by unpaired parametric t test with Welch correction (C): number of primary cilia in mIMCD3-Bbs10⁻/⁻ cells (n = ) compared to wild-type cells. Immunofluorescence staining of 24- and 48-h serum-starved cells (wild type vs Bbs10⁻/⁻ cells) with antibodies against Arl13b (primary cilium, red), γ-tubulin (basal body, green) and DAPI (DNA, blue in merge). Magnifications area shown in white boxes, white scale bars 5 μm (D): significant differences in the number of cilium presenting cells were observed, with a decrease of ciliated cells in Bbs10⁻/⁻ condition both at 24 and 48 h of serum starvation (p < 0.05 [χ²-test], white scale bars 10 μm). (E) Bbs10⁻/⁻ cells (n = 18) showed a significantly increased mitotic rate compared with wild-type cells (n = 7) (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant).
loci, in mitochondrial activities/homeostasis. We show that plasmatic metabolites discriminating BBS patients with CKD have high molecular weight and a lipophilic profile. Their precursors (long chain fatty acids) accumulate in vitro into kidney tubular cells lacking Bbs10, possibly because of impaired mitochondrial activities.

CKD is an important cause of morbidity and mortality in patients with BBS. In this setting, kidney dysfunction is characterized by a wide range of severity, from urine concentrating defect to the end-stage renal disease (Zacchia et al., 2016, 2017; Caterino et al., 2018). Little is known about the exact pathomechanisms and no specific therapy is available. However, a recent meta-analysis showed that genetics may impact renal phenotype, demonstrating an association between BBS10 mutations and severe renal phenotype in patients with BBS. Our group has recently shown that urine concentrating defect is a presenting sign of kidney dysfunction, predicting eGFR decline and indicating the need for more intensified monitoring (Zacchia et al., 2021a; Marchese et al., 2020).

Figure 4. Lipid droplets in IMCD3-Bbs10−/− cells and control

(A) Representative images of mIMCD3 kidney tubular cells treated with FFA (1.5 nM) for 24 h. FFA uptake was demonstrated by Oli Red O staining (red). Nuclei were counterstained with Hematoxylin (blue).

(B) greater magnification of mIMCD3 cells after FFA treatment demonstrating the accumulation of FFA droplets in Bbs10−/− cells. Wild-type cells are not shown as they do not accumulate FFA.

(C) quantification of FFA droplets after treatment. Three independent observers quantified the amount of lipid droplets in the cells giving a score from 0 (no cells with droplets) to 2 (all cells showing droplets). For each condition, the average score among blinded observers was considered. Barplot represents mean ± SEM of experimental triplicates. Scalebar = 50micron (A), 10micron (B).
Figure 5. Mitochondrial membrane potential and mitochondrial α-CS in mIMCD3-Bbs10 −/− cells
(A) wild type (n = 6) and Bbs10 −/− cells (n = 18) are stained with mitotracker red CMXros that are sensible to membrane potential (in red) and with citrate synthase fluorescent antibody that marks all mitochondria and is an indicator of mitochondrial biomass (in green) (white scale bar 10 μm, objective 63×). Cells 24 h after seeding are incubated with medium contained mitotracker red CMXros at a final concentration of 200 nM for 40 min at 37°C. After incubation, cells...
The present study analyzes for the first-time plasma metabolic fingerprinting of patients suffering from BBS by using a targeted approach on plasma samples, a tool that has been shown to provide mechanistic insights underlying the pathogenesis of several disorders, including kidney diseases (Liu et al., 2010).

The study revealed a clear separation of plasma metabolite profile of BBS patients with and without eGFR decline; similar results were obtained in a control group, including healthy subjects and CKD due to other causes. Interestingly, several molecules discriminating individuals with declined eGFR were shared among BBS and controls, suggesting that CKD strongly affected metabolic plasma composition. The VIP metabolites shared between patients with BBS and controls are known to be associated with CKD and are included in the group of molecules known as uremic toxins (Perna et al., 2016, 2019).

At variance, 10 metabolites appeared linked to the eGFR decline only in patients with BBS, indicating a BBS-specific effect. These metabolites have high molecular weight, lipophilic physical property, and include several phosphatidylcholines and acylcarnitines. Unfortunately, given the high genetic heterogeneity of our BBS cohort, the study is not powered enough to show possible correlations between metabolites and specific genotypes; thus, the analysis was conducted in the entire BBS cohort and the results were compared with non-BBS individuals.

As plasmatic phosphatidylcholine are major components of plasma lipoproteins, one possible explanation is that abnormal phosphatidylcholine plasma levels reflect dyslipidemia, a common finding in patients with BBS. This hypothesis required further analysis. The specific relationship of three acylcarnitines with the eGFR in patients with BBS attracted our attention.

Carnitine and its esters are detected in all biological fluids, including plasma. As long-chain-fatty-acid are impermeable to mitochondrial membranes, carnitine serves as a shuttle for their transport to the mitochondria (Costanzo et al., 2017).

The kidneys are highly metabolic organs that use high levels of ATP to exert their functions, especially tubular reabsorption. Mitochondrial fatty acid oxidation (FAO) serves as the preferred source of energy production (Liu et al., 2010; Zacchia et al., 2018). The excess fatty acids that are not catabolized by mitochondria are esterified with glycerol and deposited as lipid droplets. To shed light on abnormal acylcarnitine levels in BBS patients with kidney impairment, we used an in vitro model of BBS, renal-derived epithelial cell line (IMCD3) lacking Bbs10, as the latter is one of the major BBS genes correlating with kidney dysfunction in patients with BBS, according to the literature (21). The model was developed using the CRISP/CAS9 approach, which allowed a dramatic reduction of Bbs10 genetic transcription; unfortunately, the low endogenous Bbs10 protein abundance in IMCD3 cells hindered its quantification in both wild type and depleted cells, at protein levels. However, our results showed that experimental cells displayed metabolic aberrations, characterized by intracellular lipid accumulation and mitochondrial dysfunction. To further validate the cellular model, we have analyzed the presence of cellular inclusions using Red-oil staining in the urine sediment of patients with BBS10 mutation. Our results support the presence of lipids droplets in tubular cells of the urine sediment of patients with BBS10.

Increased lipid accumulation is well described in several experimental models of nephropathies, including acquired metabolic kidney disorders (obesity and diabetic nephropathy), acute and chronic kidney disease, and even genetic disorders (Thongnak et al., 2020). It is debated whether lipid accumulation is the...
cause or the result of kidney injury. It is believed that increased intracellular lipid deposit is the effect of an imbalance between lipid production/uptake and lipid catabolism. Altered mitochondrial function is considered a cause of renal lipid drops accumulation in diabetic nephropathy.

We found signs of mitochondrial dysfunction in IMCD3 cells lacking Bbs10, as shown by reduced potential membrane and citrate synthase staining. Whether mitochondrial dysfunction may be the cause or the effect of lipotoxicity was unclear. However, we performed the analysis of BBS10 interactors, using a functional proteomics approach, to provide further insights into the link between Bbs10 depletion, lipid cellular accumulation, and mitochondrial dysfunction. Interestingly, we found that BBS10 interactors, in vitro, included six mitochondrial proteins. It’s intriguing to note the known BBS10 interactors, BBS6 and BBS12, were not found in our analysis. Unfortunately, mass spectrometry methods were unable to detect poorly abundant proteins and/or ligands weakly interacting with BBS10. In fact, the interaction between chaperonin-like BBS proteins has been demonstrated by alternative methods (Seo et al., 2010).

The interaction of BBS10 with several mitochondrial proteins prompted us to better analyze its subcellular localization. For the first time, we showed that in HEK cells over-expressing human BBS10, the protein localizes in all cellular compartments, cytoplasm, nucleus, and mitochondria. To corroborate this finding, we studied BBS10 protein levels in renal biopsy samples of individuals with proteinuria, that did not show significant abnormalities in kidney structure. BBS10 protein was detected in tubular cells; within the cytoplasm it showed a colocalized a marker of mitochondria, confirming in humans its extra-basal body localization. However, BBS10 has no known target sequence for mitochondria, according to DeepMito software. Therefore, the molecular interaction of BBS10 and the mitochondrial proteins found by immune-precipitation must be interpreted with caution. As immunoprecipitation destroys the subcellular localization of proteins, BBS10 might artificially arrive in contact with proteins that would be sequestered in subcellular compartments. Among the interactors described in Table 4, the BAG2 protein is expressed on the outer mitochondrial membrane and therefore might physically interact with BBS10.

| Gene ID | Protein ID | Function |
|---------|-----------|----------|
| BAG2    | O95816    | Mitochondrial clearance through the recruitment of PARKIN by the stabilization of PINK1 on the outer membrane of depolarized mitochondria. Bcl2-associated athanogene 2 (BAG2) promotes mitophagy and physically interact with PINK1 (Qu et al., 2015) |
| TUFM    | P49411    | Homeostasis of parkin-dependent mitophagy regulation (Lin et al., 2020) |
| MTHFD1L | Q6UB35    | Folate-mediated one-carbon (1C) metabolism pathway (Xiu and Field, 2020) |
| ACAT1   | P24752    | It is involved in beta-oxidation. The deficiency in this enzyme is associated with an increased amount of cholesterol compounds. Its deregulation in CKD is associated to lipids accumulation (Cho et al., 2010) |
| GOT2    | P00505    | Facilitates cellular uptake of long-chain free fatty acids |
| TIMM50  | Q3ZCQ8    | It is an essential component of the TIM23 complex, the mitochondrial inner membrane machinery that imports cytosolic proteins into the mitochondrial inner compartment. TIMM50 is essential for cell survival in OxPhos-dependent conditions (Reyes et al., 2018) |
As stated above, the link between mitochondrial dysfunction and CKD is an intriguing and emerging paradigm (Bhatia et al., 2020). In the last years, the phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) and PARKIN have been shown to participate in a signaling pathway governing mitochondrial quality control (Valente et al., 2004, O’flanagan and O’neill, 2014). The PINK1/Parkin signaling activates the ubiquitination of outer mitochondrial membrane proteins to promote priming dysfunctional mitochondria for lysosome degradation pathways. Interestingly, Bcl2-associated athanogene 2 (BAG2), one of the BBS10 interactors in our study, was identified as a mediator promoting mitophagy by inhibiting PINK1 ubiquitination and degradation (Luciani et al., 2020; Qu et al., 2015). If BBS10 is required for correct BAG2 function, we could expect abnormalities in cleaning cells from damaged mitochondria. Our findings showed a decreased mitochondrial membrane potential in cells lacking Bbs10. Moreover, immunoblotting studies revealed an increased abundance of Vdac1 compared to control cells. Our data suggest that Bbs10 deficiency may lead to the cellular accumulation of dysfunctional mitochondria (Figure 6).

Even though the exact link between mitochondrial dysfunction/increased intracellular lipid accumulation/and kidney impairment should be better deciphered, as well as the direct role of BBS10 depletion in mitochondrial clearance, this study shows for the first time that CKD in patients with BBS is characterized by metabolic abnormalities, including both common features of CKD and specific BBS abnormalities; these alterations could be the result of a specific role of BBS proteins on renal epithelial mitochondrial homeostasis, at least in the setting of Bbs10 depletion.

Limitation of the study
The study has some limitations. The patients’ cohort is quite heterogeneous at genetic levels: the selection of BBS10-mutated patients would reduce the patients’ number, affecting the statistical power of our analysis. Moreover, CRISPR/Cas9 technology brings its own limitation, including the difficulty to exclude
off-target effects. Future experiments might also take into consideration rescue experiments, which are exceedingly difficult in this cellular model. Finally, further studies are needed to elucidate the exact mechanisms linking Bbs10 invalidation/mitochondrial abnormalities/BBS10 interaction with mitochondria-related proteins.

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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105230.

ACKNOWLEDGMENTS
This work is generated within the European Reference Network for Rare Kidney Diseases (ERKNet). We acknowledge the Italian Association of BBS patients’ families (ASBBI) for its support. We acknowledge Alessia Romano of the Ceinge the Advanced Light Microscopy (ALM) Facility for expert help in data acquisition.

AUTHOR CONTRIBUTIONS
EM, MC, DV, AC, and NG performed the experiments. MZ designed the experiments and collected the data from human subjects. GC coordinated the in vitro and in vivo experiments. MR and MC analyzed and validated metabolomics studies. VDI, FS, LD, and ST recruited patients. DV performed the statistical analysis. AC and RF assisted in vitro studies. FDVB and VN performed genetic analysis. All authors participated in the drafting and critical review of the article.

DECLARATION OF INTERESTS
The authors declare no competing interests.
INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

Received: July 22, 2021
Revised: February 28, 2022
Accepted: September 23, 2022
Published: November 18, 2022

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Zacchia, M., Capolongo, G., Trepiccione, F., and Marion, V. (2017). Impact of local and systemic factors on kidney dysfunction in Bardet-Biedl syndrome. Kidney Blood Press Res. 42, 784–793.

Zacchia, M., Marchese, E., Trani, E.M., Caterino, M., Capolongo, G., Perna, A., Ruoppolo, M., and Capasso, G. (2020). Proteomics and metabolomics studies exploring the pathophysiology of renal dysfunction in autosomal dominant polycystic kidney disease and other ciliopathies. Nephrol. Dial. Transplant. 35, 1853–1861.

Zacchia, M., Zacchia, E., Zona, E., Capolongo, G., Raiola, I., Rinaldi, L., Trepiccione, F., Ingrasso, D., Perna, A., Di Iorio, V., et al. (2016). Renal phenotype in Bardet-Biedl syndrome: a combined defect of urinary concentration and dilution is associated with defective urinary Aqp2 and Umod excretion. Am J. Physiol. Renal. Physiol. 311, F686–F694.

Zaghloul, N.A., and Katsanis, N. (2009). Mechanistic insights into Bardet-Biedl syndrome, a model ciliopathy. J. Clin. Invest. 119, 428–437.
## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| **Antibodies**       |        |            |
| Arl13b               | Proteintech | Cat# 17711-1-AP RRID:AB_2060867 |
| γ-tubulin            | Sigma-Aldrich | Cat# T6557 RRID:AB_477584 |
| Citrate synthase     | Abcam | Cat# ab96600 RRID:AB_10678258 |
| Tubulin              | Sigma-Aldrich | Cat# T6074 RRID:AB_4775 |
| Flag M2              | Sigma-Aldrich | Cat# F1804 RRID:AB_262044 |
| Vdac1                | Millipore | Cat# AB10527 RRID:AB_10806766 |
| **Biological samples** |        |            |
| mlMCD3               | ATCC | Cat# CRL-2123 RRID:CVCL_0429 |
| Hek293T              | Ceinge Institute | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Lipofectamine 2000   | Thermo Fisher Scientific | Cat# 11668030 |
| Oil Red O powder     | Sigma-Aldrich | Cat# O-0625 |
| Mitotracker Red CMXRos | Thermo Fisher Scientific | Cat# M7512 |
| Mowiol 4-88 Reagent  | MilliporeSigma | Cat# 475904100GM |
| SuperScript™ VILO™ MasterMix | Thermo Fisher Scientific | Cat# 11756050 |
| Anti-FLAG® M2 Magnetic Beads | Sigma-Aldrich | Cat#M8823 |
| EUROGOLD Trifast     | EuroClone | Cat# 961590054 |
| **Critical commercial assays** |        |            |
| FlexiGene DNA Kit (250) | Qiagen | Cat# 51206 |
| Absolute p180 kit    | Biocrates life | Cat# 0073.6 |
| S-Trap™ micro        | Protifi | N/A |
| Qproteome Cell Compartment Kit | Qiagen | 37502 |
| **Recombinant DNA**  |        |            |
| Bbs10 CRISPR/Cas9 KO Plasmid (m) | Santa Cruz | Sc-428052 |
| Bbs10 HDR plasmid (m) | Santa Cruz | Sc-428052-HDR |
| Control CRISPR/Cas9 Plasmid | Santa Cruz | sc-418922 |
| BBS10 cDNA ORF Clone, Human, N-DYKDDDDK (Flag®) tag | Sino Biological | HG29785-NF |
| **Software and algorithms** |        |            |
| Perseus software version 1.6.0.7 | www.perseusframework.org, MPI of Biochemistry, Martinsried, Germany | N/A |
| MaxQuant software version 1.6.5.0 | www.maxquant.org | N/A |
| R software           | R i386 4.1.3 | N/A |
| **Other**            |        |            |
| nanoRSLC-Q Exactive PLUS (RSLC Ultimate 3000) | Thermo Fisher Scientific | N/A |
| Triple Quad™ 5500+ System QTrap-Ready | ABSciex | N/A |
| Agilent 1260 Infinity II HPLC | Agilent | N/A |
RESOURCE AVAILABILITY

Lead contact
The raw data and other study materials will be provided after request to the corresponding author, Prof. D. Viggiano.

Materials availability
This study did not generate new and unique reagents.

Data and code availability

- Data used in statistical analyses have been deposited in the paper’s supplemental information and are available as of the date of publication.
- All original codes are available in the paper’s supplemental information.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Subject enrollment
Patients referring to the ambulatories of rare kidney and ocular disorders of the University of Campania, L.Vanvitelli with a clinical diagnosis of BBS according to Beales criteria (Beales et al., 1999) were enrolled in the study. Kidney function was evaluated estimating the GFR (eGFR) using the CKD-Epidemiology Collaboration Equation (CKD-EPI), which considers serum creatinine levels, age, gender and race (Levey et al., 2009). Given the young age of patients, chronic kidney disease (CKD) was defined as an eGFR lower than 90 mL/min/1.73 m². Post-axial polydactyly was defined as the presence of an extra-finger; retinal dystrophy was assessed after ocular examination; obesity was defined as body max index over 30 kg/m². Intellectual disability referred to defect in writing, spelling, speaking or memories/social/coordination defects.

Clinical (age, gender, comorbidities) and anthropometric parameters (height, weight and body mass index) were investigated at basal visit.

Renal function was assessed as follows: the glomerular filtration rate (eGFR) was estimated using CKD-EPI formula or Schwarts formula in children (<15 years). Serum creatinine was measured with an enzymatic method on an autoanalyzer, and the values were rounded to the second decimal point. Maximal urine concentration ability was assessed on second void urine sample, collected after over 12 h fasting and dehydration; urine osmolality was measured by a freezing point depression osmometer (model 3320 osmometer, Advanced Instruments, Inc.) as detailed elsewhere(Caterino et al., 2018; Zacchia et al., 2016).

Study approval
This study is in full compliance with the Declaration of Helsinki. Human studies have been approved by the Institutional Review Boards of the University of Campania L. Vanvitelli (N 76, 24/02/2014). Written informed consent was received from participants prior to inclusion in the study. Participants have been identified by number, not by name.

METHOD DETAILS

Genetic analysis
All patients underwent genetic analysis using Next Generation Sequencing (NGS) technology. Written informed consent was obtained from all participants; genomic DNA was extracted from whole blood sample with the FlexiGene DNA Kit (Qiagen) following the manufacture’s instruction. Patient DNA analysis by NGS was performed with the Illumina MiSeq System, as described previously (20). The variant frequency was analyzed with the ExAC database and in silico prediction tools, including PolyPhen and SIFT, were used to assess pathogenicity. Putative pathogenic variants were confirmed by Sanger sequencing (3130 Genetic Analyzer Applied Biosystems), according to the manufacturer’s protocol. Genotype-phenotype analysis was elucidated after consultation of specific Databases as Pubmed, ClinVar and OMIM. Each new variant was analyzed for inheritance and parental segregation.
Serum collection and preparation
Blood samples were obtained after an overnight fast. Blood was centrifuged (10 min, 2000 g at 4°C) and plasma was aliquoted into separate polypropylene tubes that were immediately stored at −80°C. One 10-μL aliquot was analyzed with the Biocrates AbsoluteIDQ p180 kit (Biocrates Life Science AG, Innsbruck, Austria), as recommended by the manufacturer and analyzed on a Triple Quad 5500 + System QTrap-Ready (AB Sciex) coupled with an Agilent 1260 Infinity II HPLC equipped with an Agilent C18 HPLC column.

Targeted metabolites identification and quantification
The Biocrates AbsoluteIDQ p180 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) is a mass spectrometry-based metabolome platform that allows the identification and quantification more than 180 metabolites, combining a flow injection analysis (FIA) and a liquid chromatography (LC) method. It allows us to measure metabolite, including amino acids, biogenic amines, acylcarnitines, lysophosphatidylcholines, phosphatidylcholines, sphingomyelins and the sum of hexoses (one resultant metabolite) (Caterino et al., 2021; Imperlini et al., 2016). Serum sample preparation was carried out according to the manufacturer’s protocol (Biocrates, User Manual-P180). Briefly, 10 μL of sample (serum sample, quality controls, zero samples, or calibrators) were added onto the filter inserts and dried for 30 min under a nitrogen stream. Quantification was carried out using internal standards (ISTD) and a calibration curve (Cal 1 to Cal 7). Three human plasma samples spiked with different concentrations of reference analytes (QC1-3) were analyzed as quality control, according to manufacturer’s protocol. Metabolites and their abbreviations were summarized in Table S1. Amino acids and biogenic amines were derivatized for 20 min with an excess of 5% phenylisothiocyanate in ethanol/water/pyridine (ratio 1/1/1, v/v/v), and subsequently dried for 45 min under a nitrogen stream. Metabolites and internal standards were then extracted with 300 μL methanol containing 5 mM ammonium acetate by shaking for 30 min and eluted by centrifugation for 5 min at room temperature and 450 rpm. From the eluate 50 μL was diluted with 450 μL of 40% methanol (50/50, v/v in HPLC grade water) for the LC-MS/MS analysis, and 10 μL of the eluate was diluted with 490 μL of FIA kits’ running solvent (1/5, v/v) for the FIA run.

For the FIA analysis the injection volume was 20 μL with a solvent mobile at an initial flow rate of 0.03 mL/min until 1.6 min, followed by 0.20 mL/min for 1.6 min and 0.02 mL/min for 0.20 min. The auto sampler was cooled at 10°C. The ion source was operated in positive ion mode using the following parameters: spray voltage 55 kV, temperature of 450°C, GS1 20, GS2 40, CUR 30, CAD 8. The ion source was operated in negative ion mode using the following parameters: spray voltage 55 kV, temperature of 450°C, GS1 20, GS2 40, CUR 30, CAD 8. Data were acquired using Analyst software (version 1.7 Ab Sciex) and Concentrations of metabolites monitored by FIA were directly calculated in MetIDQ Oxygen 2976 (Biocrates Life Sciences Innsbruck, Austria).

Evaporation steps were performed using a nitrogen evaporator (Ultravap, Porvair Sciences, Leatherhead, Great Britain), and mass spectrometry analyses were conducted on using on a Triple Quad 5500 + System QTrap-Ready (AB Sciex) coupled with an Agilent 1260 Infinity II HPLC and controlled by the Analyst 1.7 software (ABSciex). Liquid chromatography was performed on a C18 column (Zorbax eclipse XDB 3 μm, 80 mm, 3.5 μm) using acetonitrile and water with 0.2% formic acid as running solvent. FIA-MS analyses was performed by using FIA kits’ running solvent as the running solvent. Finally, the molecular species quantification was carried out by MRM, Multiple Reaction Monitoring).

On each plate, three plasma samples spiked with different concentrations of reference analytes (QC1-3) and five reference plasma samples (human plasma) were run to serve as quality control and for the evaluation of plate effects, respectively. For each of the three extraction solvents, 10 μL of solvent were transferred three times onto the kit plate and analyzed with internal standards serving as zero samples. Metabolites’ concentrations were summarized in Table S2.

Generation of a cell line lacking Bbs10 using CRISPR/Cas9 technology
To obtain mIMCD3 with Bbs10 gene knockout we used by the CRISPR/Cas9 technique as previously described (Costanzo et al., 2020a). Two types of plasmidic constructs (Santa Cruz Biotechnology) were used: i) Bbs10 CRISPR/Cas9 KO Plasmid (m) and ii) Bbs10 HDR plasmid (m). The first type of construct is able to transiently express Cas9 nuclease and a single guide RNA (sgRNA) directed to an exon in the 5′ region of murine Bbs10 gene. Indeed, the Bbs10 CRISPR/Cas9 KO Plasmid (m) consists of a pool of three different plasmids which differ each other only for a sequence of 20 nucleotides of the sgRNA. In fact,
each of three sgRNA is directed to a different site (Figure S6) in the 5' region of the Bbs10 gene. Therefore, the three sgRNAs and Cas9 generate three different double-strand break (DSB) sites in the Bbs10 gene.

The second type of construct (Bbs10 HDR plasmid (m)) contains two regions which are called Arms and which have sequences that are totally homologue to the regions flanking the DSB. When Cas9 generates the DSB, the presence of HDR plasmid enable the Homology-directed Repair (HDR) and the Arms are used by the cell as DNA template for this process. The Arm that maps upstream on the sense (+) strand the DSB site is called 5' Arm, while the Arm that maps downstream the DSB site is called 3' Arm (Figure S5). Among the two Arms there is an expression cassette able to express a red fluorescent protein (RFP) and a gene which confers resistance to puromycin (Puro). Therefore, the effect of the HDR process is the insertion of such expression cassette within the DSB site. Thus, the cell clones with Bbs10 KO produce RFP and are resistant to puromycin. Since three different sgRNAs are used to generate as many DSBs, also the Bbs10 HDR plasmid (m) consists of a pool of three different plasmids which differ each other for the sequences of the Arm pairs. Each Arm pair is designed to flank one corresponding DSB site. Briefly, 1.5 x 10^5 cells/mm² of wild type cells were seeded in a 10 cm diameter plate with culture medium without antibiotics. After 24 h, the cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) as transfection reagent. 48 h after the transfection, the cells were incubated in a selective medium containing 3 μg/mL puromycin (Santa Cruz Biotechnology, Dallas, TX, USA). The culture of transfected cell (Bbs10-KO pool) was kept in the selective medium for several days with two-day changes of medium to eliminate detached cells and to preserve adherent puromycin-resistant cells. In the following weeks, the puromycin-resistant cells were detached, and re-plated after a proper dilution, in order to have separate colonies, each one deriving from a single resistant cell clone. RFP signal was used as a marker of the efficiency of the CRISPR/Cas9-mediated genome-editing. The colonies with a brighter RFP signal were then detached and kept in culture separately. Bbs10-KO pool and the clones (four Bbs10KO) were tested by qRT-PCR, in order to confirm the Bbs10 deletion.

To consider possible off-target effects of CRISPR-CAS 9 BBS10 deletion, we have exploited three guide RNAs which bind to three different sites within the same BBS10 gene (homology-directed repair, HDR). This strategy increases the efficiency of the deletion and its accuracy (see Figure S5). Furthermore, this construct has been inserted in three different cell mIMCD3 clones with different DNA insertion sites. Therefore, any off-target effect of CRISPR-CAS 9 insertion would be expected to have low chance to appear in all four clones. Control cells consisted in mIMCD3 cells transfected with Cas9 Nuclease Only. To limit clone dependent effects, four different KO clone cells were used, in addition to the negative control line transfected with Cas Nuclease Only“.

**Generation of a cell line stably expressing BBS10 for protein-protein interactions (PPIs)**

For the generation of cell line stably expressing BBS10-flag protein, 1.5 x 10^5 cells/mm² of Hek293t/17 wild type cells were seeded in a 10 cm diameter plate and kept in culture in a medium without antibiotics. After 24 h, cells were transfected by using 10 μg of BBS10 cDNA ORF Clone, Human, N-DDK (Flag) tag expression plasmid (Sino Biological, SB) and Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) following the supplier instructions. 48 h after the transfection, the cells were incubated in a selective medium containing 75 μg/mL hygromycin (Santa Cruz Biotechnology, Dallas, TX, USA). The culture of transfected cell (BBS10-flag pool) was kept in the selective medium for several days with two-day changes of medium to eliminate detached cells and re-plated after a proper dilution, in order to have separate colonies, each one deriving from a single resistant cell clone. The colonies were then kept in culture separately. BBS10-flag pool and the clones were tested by WB to verify the presence of flag tagged protein expression.

**Lipids oil red O staining**

Bbs10 depleted cells and wild type cells were treated with a mixture of Palmitic and oleic acid to a final concentration of 1nM for 24 h. Before treatment, lipids were dissolved in ethanol and conjugated with 10% BSA.

Cell Fixing: media was removed from cells and cells were gently washed twice with PBS. Paraformaldehyde (PFA, 4%) was added to each well and incubated for 30 min to 1 h.
FFA uptake was demonstrated by Oli Red O staining (red). Nuclei were counterstained with Hematoxylin (blue). To this aim PFA was removed and cells washed as needed with dH2O. Isopropanol (60%) was added to each well and incubate for 5 min. Oil red stock solution was prepared dissolving 60 mg of Oil Red O powder (sigma cod. O-0625) in 20 mL of of 100% isopropanol. To make Oil Red O Working Solution, add 3 parts of Oil Red O Stock Solution to 2 parts of dH2O, mix well. Oil Red O Working Solution was then filtered with 0.2 μm syringe filter and ready to use.

At this point, Isopropanol was discarded and replaced with Oil Red O Working Solution (sigma). Cells were incubated for 10-20 min. Oil Red O solution was kept out and cells were wash 2-5X with dH2O as needed until excess stain is no longer apparent. At the end Hematoxylin was added to cells for nuclei staining and incubated for 1 min. Hematoxylin was removed and the cells were again washed dH2O and the microscopy slides were mounted for detection with Mowiol 4-88 Reagent (Millipore, Billerica MA, Usa cod. 475,904). Under microscope, lipid droplets appear red and nuclei appear blue.

The amount of FFA droplets were quantified by three blinded independent observers who were instructed to give a score from 0 (no cells with droplets) to 2 (all cells showing droplets). For each condition the average score among blinded observers was considered. The resulting average scores in experimental triplicates were then statistically analyzed using ANOVA and planned comparisons with Student’s t test.

Mitochondrial membrane potential
Cells were seeded at a density of 2 x 10^3 cells/mm^2 in a 24-well plate. After 24 h, cells were stained with Mitotracker CMX-ROS (Ex/Em: 579/599) which accumulates in active (healthy) mitochondria with intact membrane potentials. Mitotracker CMX-ROS (200 nM) solution was pre-warm before incubating with cells for 40 min at 37°C. Cells then were washed twice in PBS and fixed in cold methanol for 10 min. Blocking was performed in PBS 0.1% Triton X-100 with Fetal Serum Bovine (FBS) at final concentration of 5%. Cells were incubated with primary antibody against Citrate synthase (ab96600, Abcam) over night at 4°C. Bound antibody were detected with 488 Alexa-Fluor-conjugated donkey anti-rabbit IgG antibody (Life Technologies). After two additional washes in PBS 0.1% Triton X-100, cells were incubated with a solution of 300 nM DAPI (Thermo Fisher Scientific, Waltham, MA, USA) in PBS for 10 min. The cells were again washed twice with PBS and the microscopy slides were mounted for detection with Mowiol 4-88 Reagent (Millipore, Billerica MA, USA cod. 475,904). Mitochondrial images were acquired with the confocal microscope LSM980 with 63× objective (NA 1.4) using Airyscan detector. Laser wavelength: 405, 488, 561 nm. Quantification of cell area (μm^2) and fluorescence intensity was performed using Zen 3.1 software.

Cilia staining and BBS10 staining in human kidney biopsies
After 48 h from seeding, the cells were starved in 0.1% of FBS medium to induce ciliogenesis. Cilia staining was performed after 24 and 48 h from starvation, respectively. Cells were then washed twice with PBS and fixed in cold methanol for 10 min. Blocking was performed in 1X PBS, 0.1% of saponin, 0.75% of BSA, 50 mM CINH4. The primary antibodies Arl13b (17711-1-AP, Proteintech) and γ-tubulin (T6557 Sigma) were diluted in the blocking solution and incubated overnight at 4°C. The secondary antibodies (488 Alexa Fluor and 594 Alexa Fluor, Thermo Fisher Scientific) were incubated for 1 h at room temperature. After two additional washes in PBS 0.1% Triton X-100, cells were incubated with a solution of 300 nM DAPI (Thermo Fisher Scientific, Waltham, MA, USA) in PBS for 10 min. The cells were again washed twice with PBS and The microscopy slides were mounted for detection with Mowiol 4-88 Reagent (Millipore, Billerica MA, USA cod. 475,904). z stack were first acquired with Cell Discoverer7(9 slices-2.72 μm), using Plan –Apochromat 20/0.95 objective and 2× tubulens optovar, to obtain high content images for quantification of cilia number. Automated analysis was performed with Zeiss Zen Intellesis module of Zen blue 3.1. Cilia count was normalized for numbers of cellular nuclei. Then, for each sample, 3 representative fields were acquired with confocal microscope LSM980 using 63× objective (NA 1.4) and Airyscan detector in order to obtain high resolution images. All results shown are representative of at least 3 independent experiments.

To analyze the colocalization of BBS10 and mitochondria in the kidney tubules, we performed immunofluorescence on kidney criosections from kidney biopsies of (non-BBS) patients that did not show significant changes in the kidney structure. Three biopsies were analyzed without significant qualitative changes among patients. For this experiment we used a double immunofluorescence staining against BBS10 (rabbit polyclonal 1:100) and methylmalonyl-CoA mutase (MUT, mouse monoclonal 1:100). The specificity of the anti-BBS10 antibody has been tested by western blot as reported in a previous published work (29). Primary
Antibodies were then visualized using anti-rabbit-Alexa 488 (green fluorescence) and anti-mouse-Alexa 594 (red fluorescence), respectively. DAPI was used as nuclear counterstaining. Sections were analyzed with a Zeiss Confocal microscope LSM 980 coupled with Airyscan module.

**Immunoprecipitation**

Lysis Buffer: 1X TBS (250 mM Tris-HCl pH 7.4, 1.40 M NaCl, 0.03 M KCl) 1 mM EDTA, 1% Triton X-100, 10% glycerol, protease inhibitors. Washing Buffer: 1X TBS (250 mM Tris-HCl pH 7.4, 1.40 M NaCl, 0.03 M KCl) 1 mM EDTA, 10% glycerol, protease inhibitors. HEK293 cells expressing BBS10-flag and the GFP-flag vector used as control were lysed with the Lysis Buffer. Total protein extracts were pre-cleared with mouse IgG agarose beads and incubated ON at 4°C with M2 anti-FLAG agarose-conjugated antibody beads (Sigma). The control experiment obtained by immunoprecipitation of GFP-flag vector transfected cells with anti-FLAG agarose beads allowed to rule out unspecific retained proteins as described. Non-retained proteins were then incubated with M2 anti-FLAG agarose-conjugated antibody beads (Sigma) overnight at 4°C. Beads were washed with washing Buffer. Retained protein complexes were eluted with 3XFLAG peptide and subjected to precipitation with methanol/chloroform. The protein mixture was vacuum-dried and resuspended in 10% SDS buffer to perform in situ protein hydrolysis with S-TrapTM micro spin column for interactors identification.

**Interactome analysis**

The protein extract from HEK293T_BBS10 and wt HEK293T (HEK293T_CTRL) cell lines or immunoprecipitated samples (IP) were resuspended in 10% SDS buffer. The samples were then reduced in 100 mM TCEP (Sigma-Aldrich, St. Louis, MO, USA) and carbamidomethylated in 50 mM iodoacetamide (Sigma-Aldrich, St. Louis, MO, USA) (Caterino et al., 2014). Aqueous phosphoric acid was added to a final concentration of 1.2%. A six-time volume of S-Trap buffer (90% aqueous methanol, 100 mM TEAB, pH 7.1) was added to protein complex. Finally, the mixtures were put on the S-Trap micro columns and centrifuged at 4000g for 30 s. Three washes were carried out by using 150 μL S-Trap binding buffer. The mixtures were digested using 3 μg of trypsin (Promega) at 47°C for 1 h. After elution, peptides were vacuum dried down and resuspended in 35 μL of 10% ACN, 0.1% TFA in HPLC-grade water prior MS analysis.

**nanoLC-MS/MS Measurements**

NanoLC-MS/MS analysis was carried out in order to process tryptic peptide mixtures from: i) extracts from HEK293T_BBS10 and wt HEK293T (HEK293T_CTRL) cell lines; ii) four independent biological replicates of BBS10-flag IP and four replicates for GFP-flag IP (Costanzo et al., 2018, 2020b). Samples were resuspended in 100 μL of 10% ACN, 0.1% TFA in HPLC-grade water. For each run, 5 μL were injected in a nanoRSLC-Q Exactive PLUS (RSLC Ultimate 3000) (Thermo Scientific, Waltham MA, USA). Peptides were loaded onto a μ-precolumn (Acclaim PepMap 100C18, cartridge, 300 μm i.d. x 5 mm, 5 μm) (Thermo Scientific), and were separated on a 50 cm reversed-phase liquid chromatographic column (0.075 mm ID, Acclaim PepMap 100, C18, 2 μm) (Thermo Fisher Scientific). Chromatography solvents were (A) 0.1% formic acid in water, and (B) 80% acetonitrile, 0.08% formic acid. Peptides were eluted from the column with the following gradient 5%–40% B (120 min), 40%–80% (1 min). At 121 min, the gradient stayed at 80% for 5 min and, at 127 min, it returned to 5% to re-equilibrate the column for 20 min before the next injection. Eluting peptide cations were ionized and analyzed using a Q Exactive PLUS mass spectrometer (Thermo Scientific, Waltham MA, USA). Tandem MS was performed by data-dependent acquisition (DDA) and the top 10 intensity precursor ions were fragmented by higher-energy collisional dissociation (HCD). The MS scan range was from 400 to 2000 m/z. The mass spectrometry parameters were set as follow in MS and MSMS scan: resolution 70,000 and 17,500; AGC (automatic gain control) target 3 × 10^6 and 1 × 10^5 counts; maximum injection time 200 and 120 ms in MS and MSMS respectively.

**Mass spectrometry raw-files data processing**

Raw data were processed using MaxQuant software version 1.6.5.0 and searched against Homo sapiens database (Swiss-Prot 07/2017) by Andromeda search engine. Mass Error Tolerance were set to 4.5 and 20 ppm for precursors and fragments, respectively. Additional search parameters: enzyme specificity: trypsin; enzyme missed cleavages: two per peptide; fixed modification: cysteines carbamidomethylation; variable modification: methionines oxidation, N-term acetylation. Match between runs was not allowed. The false discovery rates (FDRs) threshold lower than 1% on peptides and proteins. The reverse and common contaminants hits were removed from total output identified protein list. Proteins were quantified...
using MS1 peak intensity (LFQ, label free quantification value) as quantitative parameters, according to the MaxQuant label-free algorithm.

The proteome datasets of BBS10-flag and GFP-flag interactomes were analyzed with the Perseus software version 1.6.0.7 (www.perseusframework.org, MPI of Biochemistry, Martinsried, Germany). The identify BBS10 interactors purified by immunoprecipitation the following strategy was used: 1) imputation of undetectable peptides: peptides whose expression level was below the detection threshold of the method were considered having a value corresponding to the lowest LFQ intensity (among all peptides) observed in that experiment. 2) repeated t-tests were performed for each interactor, comparing the control cells (expressing only GFP-Flag) and BBS-10-Flag cells 3) to take into account the statistical effect of repeated t-tests, the False Discovery Rate (FDR) was calculated using the function p.adjust (method = “fdr”) in R environment. 4) Only interactors with FDR>0.05 were then considered 5) we have additionally selected candidate interactors on the basis of the effect size, by including only changes in expression level, \( \Delta \) (LFQ controls - LFQ BBS10_flag), lower than 1.4. The details of mass spectrometry protein identification and quantitation of BBS10 putative interactors are listed in Table S3.

In Table S3, for each identified protein in the immunoprecipitated fraction the following parameters are reported:

1. The identification codes, as Gene names, Protein IDs, and Protein names
2. The mass spectrometry quantitative parameters, as LFQ (Label Free quantification) for each biological replicate of the two conditions (CTRL and BBS 10)
3. The mass spectrometry identification parameters, as Unique peptides (number peptides), Unique sequence coverage (protein sequence coverage, calculated according to the number of peptides identified for each protein), molecular weight, MS/MS count (number of fragmentation spectra for each protein)
4. T-test p value
5. The corrected t-value considering the repeated tests (False Discovery Rate, FDR)
6. The Quantitative difference of the abundance of each protein in the compared conditions (BBS10 - CTRL)

The putative BBS10 interactors were selected among the significant proteins from Table S3, being more abundant in BBS10 interactome with respect to GFP non-specific interactome.

To estimate the BBS10-flag abundance compared to the endogenous protein, the amount of protein was measured in total extracts from HEK293T_BBS10 and wt HEK293T (HEK293T_CTRL) cell lines by mass-spectrometry-based quantitative methods. The protein abundance was estimated by using normalized MS counts (Table S4), as the ratio between the MS counts of the interest proteins and the total MS counts in the analyzed sample.

After, the list of interactors was subjected to clusterization using STRING (Search Tool for the Retrieval of Interacting Genes) online version 11, in order to evaluate the mitochondrial proteins into dataset. The cluster was evaluated by a significant interaction score as negative logarithm of the p value.

**Western Blot**

Protein sample were fractionated by a 10% SDS-PAGE, and transferred onto nitrocellulose membranes using a Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). Membranes were blocked for 2 h at room temperature with 5% milk in PBS with a 0.2% Tween 20. Each primary antibody used for WB was incubated O/N at 4°C in 5% milk in PBS with a 0.2% Tween 20.

All antibodies used in this study are commercially available and are listed below. From Abcam: Citrate synthase (ab96600, Abcam). From Sigma-Aldrich: α-tubulin (T6074) and Flag M2 (F1804). From Millipore: Vdac1 (AB10527).
Immunoblot detections were carried out using horseradish peroxidase-conjugated antibodies (Clarity Western ECL Substrate, Biorad, USA) and enhanced chemiluminescence (Clarity Max Western ECL Substrate, Biorad, USA). Signals were acquired by Biorad Chemidoc. Densitometry analysis was performed by ImageJ Fiji software.

Quantitative real-time PCR
For each qRT-PCR assay, 1.5 x 10^5 cells/mm2 of mIMCD3 cells were seeded in a 6 cm diameter plate and kept in culture in standard conditions (see above). After 24 h, cells were washed twice with PBS and total RNA was extracted from kidney cells using EUROGOLD Tri-Fast reagent (EuroClone, Paington, UK). 500 ng of RNA were reverse-transcribed using SuperScript™ VILO™ MasterMix (Thermo Fisher Scientific, Bremen, Germany). Then, qRT-PCR was carried out in a 7900 Real-Time PCR System PCR Thermal Cycler with appropriate primers using a SYBR® Select Master Mix (Applied Biosystems, Monza, Italy). Gene expression levels of Bbs10, PPAR-α, PPAR-γ, PGC1a Acox1, Cpt1 and Cpt2, were normalized to RNA polymerase II (Polr2A) and β2 microglobulin protein and calculated using the 2−ΔΔCt method. Average values from at least three independent experiments were graphically reported as relative units. Statistical significance was calculated by a two-tail unpaired t-test. The primers sequences are reported below.

Mitochondrial protein extracts
Mitochondrial extracts were obtained as elsewhere described (Clayton and Shadel, 2014). Briefly, 60 million cells, were washed twice in PBS and harvested by scraping in PBS. The cell suspension was then centrifuged at 230 RCF for 10 min at 4°C and the cell pellet was resuspended in 2.2 mL of RSB hypotonic buffer (10 mM NaCl, 1.5 mM MgCl2, 10 mM Tris-HCl pH 7.5, protease inhibitor cocktail) and incubated at 4°C for 15 min. The cell suspension was then put into a Dounce homogenizer and at least 5 strokes were applied. The homogenate was then transferred into a clean tube and 1.6 mL of 2.5X MS homogenization buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl pH 7.5, 2.5 mM EDTA, protease inhibitor cocktail) were added. The homogenate was then centrifuged at 1,300 RCF for 5 min at 4°C and the supernatant was recovered and put into a clean tube. This tube was again centrifuged at 1,300 RCF for 5 min at 4°C and the supernatant was again put into a clean tube. This last procedure (centrifugation and supernatant recovery) was performed one more time and the supernatant was then centrifuged at 17,000 RCF for 15 min at 4°C. The supernatant was again discarded and the pellet of mitochondria was resuspended in 1X MS homogenization buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl pH 7.5, 1 mM EDTA, protease inhibitor cocktail). The suspension was again centrifuged at 17,000 RCF for 15 min at 4°C and the supernatant was discarded. The pellet of mitochondria was resuspended in RIPA buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, protease inhibitor cocktail) and incubated for 20 min at 4°C under shaking. This last homogenate constituted the mitochondrial extract and was stored at −80°C.

Nucleus-Cytosol subcellular fractionation
Fractioned protein extracts containing cytosolic and nuclear proteins, respectively, were obtained using Qproteome nuclear protein kit (Qiagen Italia, Milan, Italy) as elsewhere described (Fioretti et al., 2021). Briefly, 20 millions cells were washed with PBS, harvested and then centrifuged for 5 min at 450 RCF.

| Primers | Forward 5’-3’ | Reverse 5’-3’ |
|---------|--------------|--------------|
| β2 Microglobulin | GGT CTTTCGTTGCGTTGCTCT | TATGTTCCGCTTCCATTCTC |
| Polr2a | GGTATGAATTGAAGCGATGTC | CACTCGGATCATGTTTCTGC |
| Bbs10 | CAAGTGTGTGTAGACGGGCA | CACACGCCCATTATCATCATCCTG |
| PPAR-α | TGCAAAACTTGGAATTGACAAGG | GATCAGCATACCCTGCTTGT |
| PPAR-γ | AGGATGCAAGGTGTTTTTCG | ACCCTGATGGGCTTGGAGAC |
| Cpt1 | GGTCTTCTCGGGTGCGAGTC | TCCCTCCCACCAGTCACCTAC |
| Cpt2 | CAAATGAGGAACCCCTGAGG | GATCCCTCATCGGGAAGTCA |
| Acox1 | CTTGGATGGTAGTGCCGAGAGA | TGGGCTTGGTGAAGGAGAGT |
| PGC1a | AGTCCCATACACACACCCAG | CCCCTGGGTCATTTTGGA |

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Then, they were lysed in 500 µL of hypotonic Buffer NL containing protease inhibitor solution and 0.1 M DTT. Detergent solution was added and, after a shaking, the suspension was centrifuged for 5 min at 10,000 RCF. The supernatant, containing the cytosolic proteins, was recovered and stored at −80°C. The pellet, containing the cell nuclei, was resuspended in 500 µL of Buffer NL and centrifuged again for 5 min at 10,000 RCF. The nuclear pellet was then resuspended in 50 µL of Buffer NX1 supplemented with protease inhibitor solution and incubated for 30 min under shaking. The suspension was then centrifuged for 10 min at 12,000 RCF and the supernatant was recovered and constituted the first nuclear fraction (N1) containing the nucleic-acid-binding proteins. The pellet was resuspended in 100 µL Buffer NX2 supplemented with benzonase nuclease, protease inhibitor solution and 0.1 M DTT. The suspension was incubated for 1 h under shaking and centrifuged for 10 min at 12,000 RCF. The supernatant was recovered and constituted the second nuclear fraction (N2), containing the insoluble nuclear proteins. To obtain a complete nuclear extract, the two nuclear fractions N1 and N2 were pooled together and stored at −80°C. The Qproteome Nuclear Protein Kit provided all the aforementioned buffer solutions and reagents and all the steps of the procedure were performed at 4°C.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using R software. The serum metabolome dataset was first filtered eliminating metabolites with more than 10 missing data. Subjects with BMI greater than 50 or less than 22 were excluded because the extreme body weights were accompanied by an alteration of the metabolic profile. Values with zero concentration have been replaced by the minimum positive value in the dataset of the same metabolite. The data were then log2 -transformed and scaled according to the Pareto scaling method. Creatinine was excluded from the analysis as it was used to calculate the eGFR and categorize subjects. The CKD state was coded as 0 if eGFR>90 and 1 otherwise. The Sparse Partial Least Squares Discriminant Analysis (SPLS-DA) in the mixOmics library was then performed separately on BBS subjects and non-BBS subjects, using the CKD state as factor. SPLS-DA is a supervised analysis focusing on the discrimination of the CKD states, thus allowing for relevant feature selection and graphical displays for metabolomic data. The SPLS-DA output performance was tested using the receiver operating characteristic (ROC) curves. Subsequently we computed the influence on the CKD categorization of every metabolite, retrieving the Variable Importance in Projection (VIP). VIP coefficients represent the importance of each metabolite to predict the CKD state. Metabolites with VIP score greater than 1 were then used for subsequent analysis. Therefore, two lists of metabolites with VIP score>1 were obtained, one for BBS patients and one for non-BBS patients. The two lists were then compared and only the metabolites present exclusively in BBS patients were then evaluated.

The resulting metabolites BBS-specific were then confirmed testing their correlation with the eGFR. To this aim, we used a linear mixed model with the eGFR as dependent variable and as predictors the metabolite concentration, the genotype (BBS vs non_BBS) and their interaction. Furthermore, the Pearson correlation coefficient between each metabolite and the eGFR was calculated for BBS and non-BBS populations. The differences between BBS and non-BBS regarding the calculated Pearson coefficients were tested using Fisher’s r-to-z comparison. Significance level was set at p < 0.05. Values for continuous variables are expressed as mean ± SEM.