Increased mitochondrial fragmentation in polycystic kidney disease acts as a modifier of disease progression

Laura Cassina | Marco Chiaravalli | Alessandra Boletta

Molecular Basis of Cystic Kidney Disorders Unit, Division of Genetics and Cell Biology, IRCCS-San Raffaele Scientific Institute, Milan, Italy

Correspondence

Alessandra Boletta, Molecular Basis of Cystic Kidney Disorders Unit, Division of Genetics and Cell Biology, IRCCS-San Raffaele Scientific Institute, Via Olgettina 58, Milano 20132, Italy.
Email: boletta.alessandra@hsr.it

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Abstract

Autosomal dominant polycystic kidney disease (ADPKD) is a common monogenic disorder, characterized by bilateral renal cyst formation. Multiple pathways are deregulated in cystic epithelia offering good opportunities for therapy. Others and we have previously reported that metabolic reprogramming, including alterations of the TCA cycle, are prominent features of ADPKD. Several lines of evidence suggest that mitochondrial impairment might be responsible for the metabolic alterations. Here, we performed morphologic and morphometric evaluation of mitochondria by TEM in an orthologous mouse model of PKD caused by mutations in the Pkd1 gene (Ksp-Cre;Pkd1flx/−). Furthermore, we measured mitochondrial respiration by COX and SDH enzymatic activity in situ. We found several alterations including reduced mitochondrial mass, altered structure and fragmentation of the mitochondrial network in cystic epithelia of Ksp-Cre;Pkd1flx/− mice. At the molecular level, we found reduced expression of the pro-fusion proteins OPA1 and MFN1 and up-regulation of the pro-fission protein DRP1. Importantly, administration of Mdivi-1, which interferes with DRP1 rescuing mitochondrial fragmentation, significantly reduced kidney/body weight, cyst formation, and improved renal function in Ksp-Cre;Pkd1flx/− mice. Our data indicate that impaired mitochondrial structure and function play a role in disease progression, and that their improvement can significantly modify the course of the disease.

Keywords

cellular respiration, Mdivi-1, mitochondrial fragmentation, polycystic kidney disease

Abbreviations: 2KW/BW, kidney weight/body weight; ADPKD, autosomal dominant polycystic kidney disease; BW, body weight; BUN, blood urea nitrogen; COX, cytochrome c oxidase; DBA, dolichos biflorus agglutinin; DRP1, dynamin-related protein-1; E, embryonic day; IMM, inner mitochondrial membrane; IP, intraperitoneal injection; KW, kidney weight; KSP, kidney-specific promoter; LTL, lotus tetragonolobus lectin; MAMs, mitochondria-associated membranes; MFN, mitofusin; OCT, optimal cutting temperature medium; OMM, outer mitochondrial membrane; OPA, optic atrophy 1; PC1, polycystin 1; PFA, paraformaldehyde; RCCs, respiratory chain complexes; SDH, succinate dehydrogenase; TBS, tris-buffered saline; TEM, transmission electron microscopy; TOM20, translocase of the outer mitochondrial membrane 20 protein; VDAC1, voltage-dependent anion-selective channel 1; P, postnatal day.

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1 | INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is a monogenic disease characterized by the development of large fluid-filled renal cysts that compress and destroy the surrounding healthy tissue.\(^1,2\) This alteration in tissue architecture ultimately causes kidney enlargement and progressive loss of renal function, resulting in end-stage renal disease by the age of 50 in half of the individuals.\(^1,2\) Germ line mutations in the PKD1 or PKD2 genes, encoding for Polycystin 1 (PC1) and 2 (PC2) proteins, account for about 85 and 10% of ADPKD cases, respectively.\(^1,2\) Loss-of-function of PC1 or PC2 induces deregulation of several signaling pathways and recent findings indicate that the alteration in cellular metabolism is a hallmark of the disease.\(^1-7\) The remodeling of glucose utilization for energy production and a wide rewiring of metabolic reprogramming of energetic pathways have been reported in mouse Pkd1 mutant cells and kidneys.\(^3,5,8\) Reduction in mitochondrial oxygen consumption rate and alterations in mitochondrial structure have also been described.\(^5,8-11\) These alterations correlate in human and mouse ADPKD cells with increased expression of miR-17, which negatively modulates mitochondrial function, through repression of the master regulator of mitochondrial biogenesis, PPARalpha.\(^12\) Moreover, PC1 and PC2 proteins localize also in the mitochondria-associated membranes (MAMs), where they can mediate calcium signaling from the ER lumen to the mitochondria matrix thus modulating mitochondrial metabolism.\(^7,9,13\) Of note, a cleaved carboxyl-terminal fragment of PC1 has been proposed to accumulate in mitochondria, indicating that PC1 may directly modulate mitochondrial function.\(^11\)

To clarify whether the defects in mitochondrial structure and function are observed in cystic tissues and could account for metabolic rewiring and contribute to disease progression, we analyzed for the first time the mitochondrial structure and function are observed in cystic tissues and could account for metabolic rewiring and contribute to disease progression, we analyzed for the first time the mitochondrial structure and function are observed in cystic tissues and could account for metabolic rewiring and contribute to disease progression, we analyzed for the first time the mitochondrial structure and function are observed in cystic tissues and could account for metabolic rewiring and contribute to disease progression, we analyzed for the first time the mitochondrial structure and function are observed in cystic tissues and could account for metabolic rewiring and contribute to disease progression, we analyzed for the first time the mitochondrial structure and function are observed in cystic tissues and could account for metabolic rewiring and contribute to disease progression, we analyzed for the first time the mitochondrial structure and function are observed in cystic tissues and could account for metabolic rewiring and contribute to disease progression, we analyzed for the first time the mitochondrial structure and function are observed in cystic tissues and could account for metabolic rewiring and contribute to disease progression, we analyzed for the first time the mitochondrial structure and function are observed in cystic tissues and could account for metabolic rewiring and contribute to disease progression, we analyzed for the first time the mitochondrial structure and function are observed in cystic tissues and could account for metabolic rewiring and contribute to disease progression, we analyzed for the first time the mitochondrial structure and function are observed in cystic tissues and could account for metabolic rewiring and contribute to disease progression, we analyzed for the first time the mitochondrial structure and function are observed in cystic tissues and could account for metabolic rewiring and contribute to disease progression.
genotypes once daily for three consecutive days, from P6 to P8. Mice were sacrificed 1 hour after the last IP injection, at P8.

2.3 | Histological analysis, immunohistochemistry, and immunofluorescence

For histological analysis mice were sacrificed at the indicated time, kidneys were collected, weighted, and fixed overnight in 10% of natural-buffered formalin (Bio-Optica, Milan, Italy). Then, samples were incubated in 70% of ethanol (Sigma-Aldrich, St. Louis, MO, US). After passages in ethanol ascending scale up to 100%, samples were embedded in paraffin blocks.

Formalin-fixed paraffin-embedded consecutive sections (4 μm) were dewaxed and hydrated through graded decrease alcohol series and stained. For histological analysis in bright-field microscopy, slides were stained using standard protocols for Hematoxylin and Eosin (using Mayer’s Hematoxylin, #05-06002/L and Eosin, #05-10002/L, Bio-Optica, Milan, Italy). Images were acquired using Zeiss AxiosImager M2m (Zeiss, Oberkochen, Germany).

For immunofluorescence analysis, after euthanasia kidneys were removed, washed in 1x PBS, weighed, and fixed in 4% of paraformaldehyde (PFA). After incubation in a sucrose-in-PBS gradient scale from 10% to 30%, samples were incubated in 10% of glycerol (Sigma-Aldrich, St. Louis, MO, US) in a mixture of optimal cutting temperature medium (OCT) (Bio-Optica, Milan, Italy) and 30% of sucrose, and then, embedded in OCT. About 5 μm kidney sections were washed in 1x PBS, fixed for 10 minutes in 4% of PFA, and permeabilized in 0.2% of Triton X-100 (Sigma-Aldrich, St. Louis, MO, US), in 1x PBS. Sections were blocked in 3% of BSA (Sigma-Aldrich, St. Louis, MO, US) 1x PBS for 1 hour at RT, incubated over night at 4°C with Translocase of The Outer Mitochondrial Membrane 20 protein (TOM20) primary antibodies diluted in blocking solution with 0.1% of Triton, washed with 1x PBS. Secondary antibodies (Goat anti-Rabbit IgG (H + L) Cross-Adsorbed, Alexa Fluor 647 (#A-21244 from Thermo Fisher Scientific, Waltham, MA, US) were incubated with DBA for one hour in blocking solution 0.1% of Triton X-100, nuclei were stained with DAPI, and slides were mounted with Fluorescence mounting medium (Dako, Agilent Technologies, Santa Clara, CA, US). Images were acquired using GE healthcare DeltaVision Ultra microscope (GE Healthcare, Chicago, IL, US). Representative images in Figure 4 are Z projections of 20 image stacks.

For immunohistochemistry staining, PFA-fixed OCT-embedded kidney consecutive sections (5 μm) were immunostained for Biotinylated DBA (Cat B-1035, Lot W0725, Vector Laboratories, San Diego, CA, US) according to manufacturer’s procedure. Any endogenous peroxidase activity were quenched by incubating the slides with 0.3% of H₂O₂ in methanol for 10 minutes at RT. Primary antibody was used at dilution of 1:800, 1h RT, and developed with Streptavidin-HRP diluted 1:1000 (cat 554066, Lot 30882, BD Biosciences (Bedford, MA, US). After immunostaining, DAB substrate-chromogen was applied to sections for 5 minutes at RT and counterstained with Mayer’s hematoxylin, dehydrated, and mounted with Eukitt (#09-00100, Bio-Optica, Milan, Italy). Proper external positive and negative controls were run simultaneously. Images were acquired using Zeiss AxiosImager M2m (Zeiss, Oberkochen, Germany).

2.4 | COX and SDH staining

Kidneys were collected from mice of the indicated genotypes at P4, weighted, and embedded directly in OCT. Cryostat 8 μm serial kidney sections were rehydrated with 1x PBS and in situ activity staining for cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) enzymes were performed used kits from Bio-Optica, Milan, Italy (COX stain #30-30115LY; Succinic Dehydrogenase stain #30-30114LY) according to manufacturer’s instructions. The slide sections were counterstained for 5 minutes with Hematoxylin Solution, Harris Modified solution (1:10 in distilled water; Bio-Optica, Milan, Italy) and mounted with the indicated mounting medium. Images were acquired using Zeiss AxiosImager M2m (Zeiss, Oberkochen, Germany).

2.5 | TEM imaging

Kidneys were collected from mice of the indicated genotypes at P4 (N = 3, Ksp-Cre;Pkd1floxed/ or Pkd1floxed/+, interchangeably and N = 3, Ksp-Cre;Pkd1floxed/−), or at P8, after treatment with vehicle or Mdivi-1, (N = 2, Ksp-Cre;Pkd1floxed/ or Pkd1floxed/+), interchangeably and N = 2, Ksp-Cre;Pkd1floxed/−). Mice were weighted, and fixed for 24 hours at 4°C with 4% of PFA and 2.5% of glutaraldehyde in 125 mM of cacodylate buffer. Samples were then postfixed for 1 hour with 2% of OsO4 in 125 mM of cacodylate buffer, washed, and embedded in Epon. Conventional thin sections (60 nm) were collected on uncoated grids, stained with uranyl and lead citrate, and examined with Leo912 80kv Transmission Electron Microscope (Zeiss, Oberkochen, Germany).

2.6 | Image analysis

Morphometric analysis of mitochondria in TEM images was performed on 30 images of kidney sections from 3 control and 3 Ksp-Cre;Pkd1floxed/− kidneys. Mitochondrial parameters were measured using the freehand line selection tool
of ImageJ software (NIH, https://imagej.nih.gov/ij/) to calculate mitochondria area and perimeter, major and minor axis, circularity, and roundness. In particular, roundness is the inverse of the aspect ratio (major axis/minor axis) and is calculated as follows: $4 \times \text{area}/\pi \times \text{major_axis}^2$. Circularity is $4\pi \times \text{area}/\text{perimeter}^2$. A value of 1.0 indicates a perfect circle. For both, as the values approach 0.0, it indicates an increasingly elongated shape.

Densitometric analysis of western blot bands from three independent experiments was performed using ImageJ software, followed by Student's $t$ test analysis.

### 2.7 Western blot analysis

For western blot analysis deep-frozen kidneys are mechanically smashed and homogenized using cold lysis buffer solution (150 mM NaCl, 20 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, 10% of glycerol, 1% of Triton X-100, pH 7.2) supplemented with complete protease inhibitor cocktail (Roche, Basel, Switzerland) and phosphatase inhibitors (1 mM glycercophosphate, 1 mM sodium orthovanadate, and 1 mM sodium fluoride). Total protein extracts were quantified with Biorad Protein Assay (Biorad, Hercules, CA, US) and Laemmli buffer was added to the samples. Proteins were resolved in 4%-12% of Tris-Glycine gradient gels (Life Technologies, Carlsbad, CA, US) or standard 8% of SDS-PAGE, and transferred onto nitrocellulose membranes (Millipore, Merck KGaA, Darmstadt, Germany). Membranes are blocked in 1x Tris-buffered saline, 0.1% of Tween-20 (TBS-T) with 5% of skim milk. Primary antibodies for western blot analysis were diluted in 1x TBS-T supplemented with 3% of BSA (#A7906, Sigma-Aldrich, St. Louis, MO, US) and Laemmli buffer was added to the samples. Proteins were resolved in 4%-12% of Tris-Glycine gradient gels (Life Technologies, Carlsbad, CA, US) or standard 8% of SDS-PAGE, and transferred onto nitrocellulose membranes (Millipore, Merck KGaA, Darmstadt, Germany). Membranes are blocked in 1x Tris-buffered saline, 0.1% of Tween-20 (TBS-T) with 5% of skim milk. Primary antibodies for western blot analysis were diluted in 1x TBS-T supplemented with 3% of BSA (#A7906, Sigma-Aldrich, St. Louis, MO, US). HRP-conjugated secondary antibodies were diluted 1:10.000 (or more if necessary) in 5% of skim milk, 1x TBS-T, and detected by ECL (#RPN2106, GE Healthcare, Chicago, IL, US) alone or supplied with 10% of SuperSignal West Femto (#34095, Thermo Fisher Scientific, Waltham, MA, US) when required.

### 2.8 Mitochondria respiration assays

Oxygen consumption rates (OCR) were measured in mouse cortical collecting duct cells (mCCD) after over-night treatment with 25, 75 μM of Mdivi-1, or vehicle by SeaHorse XFe96 Analyzer (Agilent Technologies, Santa Clara, CA, USA) using SeaHorse XF Cell Mito Stress Test, following the manufacturer’s instructions. Briefly, the day before the assay, mCCD cells were counted and plated in 96-well SeaHorse cell culture microplates at a density of 15 000 cells per well, in mCCD medium (Growth medium was DMEM-F12 supplemented with 5 μg/mL of insulin, 60 nM of selenium, 5 μg/mL of transferrin, 5 ng/mL of mouse EGF, 1 nM of triiodothyroidine, 50 nM of dexamethasone, and 2% of FCS), and incubated in a 5% CO$_2$ incubator at 37°C overnight. The following day, culture medium was replaced with Agilent Seahorse XF DMEM Medium pH 7.4 supplemented with 10 mM glucose (Sigma-Aldrich), 1 mM sodium pyruvate (Gibco), and 2 mM L-glutamine (Gibco) for Mito Stress Test assay. The plate was incubated at 37°C for 1 hour in a non-CO$_2$ incubator, and then, we performed Mito Stress Test. After OCR baseline measurements, oligomycin A (O), FCCP, and rotenone/antimycin A (R/A) were added sequentially to each well at the final concentrations of 1, 1.5, and 0.5 μM, respectively. Results were normalized by cell viability, using CyQUANT Cell Proliferation Assay (Thermo Fisher Scientific). Data are expressed as pmol of oxygen per minute per arbitrary units. Basal respiration, ATP production, proton leak, and maximal respiration were calculated using Seahorse XF Mito Stress Test Report Generator (Agilent Technologies).

OCR was also measured in mitochondria isolated from kidneys from control and cystic mice treated with vehicle or Mdivi-1 by SeaHorse XFe96 Analyzer, as previously described. Briefly, mice were sacrificed and kidneys were immediately extracted and placed in ice. Crude mitochondria extracts were prepared by tissue homogenization in mitochondrial isolation buffer (210 mM of d-Mannitol, 70 mM of sucrose, 5 mM of HEPES, 1 mM of EGTA, and 0.5% of fatty acid-free BSA pH to 7.2). Mitochondria pellets were resuspended in mitochondrial assay solution (220 mM of d-Mannitol, 70 mM of sucrose, 10 mM of KH$_2$PO$_4$, 5 mM of MgCl$_2$, 2 mM of HEPES, 1 mM of EGTA, and 0.2% of fatty acid-free BSA, pH to 7.2) supplemented with 10 mM of Glutamate and 5 mM of Malate for complex I-driven respiration.

Respiration by the mitochondria (4 μg/well) was sequentially measured in a coupled state with substrate present (basal respiration, state 2), followed by state 3 (phosphorylating respiration, induced with the addition of 2 mM ADP), state 4o (induced with the addition of 6 μM oligomycin), and then, maximal uncoupler-stimulated respiration (state 3u, induced with the addition of 4 μM FCCP). Non-mitochondrial respiration was induced with the addition of 8 μM antimycin A.

We calculated respiratory states as follows: basal respiration by subtracting the OCR values at minute 40 from the values at minute 1; state 3 by subtracting the OCR values at minute 40 from the those at minute 12; state 4o by subtracting the OCR values at minute 40 from the values at minute 20; state 3u by subtracting the OCR values at minute 40 from the values at minute 30. We calculated the respiratory control ratio (RCR), the index of mitochondrial coupling, by dividing the corrected values of state 3/state 4o. Data are expressed as pmol of oxygen per minute per μg proteins. Mitochondrial preparations and ER contaminants were analyzed by western blot (Figure S4).
2.9 | Statistics

Student’s t test, Chi-squared test, and two-sample Kolmogorov-Smirnov test were used for significance calculation and p values are indicated in the figure legends. GraphPad Prism 8 software, and Matlab® software were used as statistical analysis tools.

2.10 | Original data

The original TEM images used for quantification of the morphology of mitochondria and the scans of the original western blots are available through Mendeley at the following link: https://doi.org/10.17632/dn3c6k76cy.1.

3 | RESULTS

3.1 | Reduced mitochondrial mass and aberrant mitochondria in cyst-lining epithelial cells of Pkd1 mutant mice

Metabolic reprogramming has been described as an important feature of ADPKD.¹⁻⁷ In particular, we previously found increased glycolysis and TCA cycle metabolic reprogramming in an orthologous mouse model of PKD.³⁻⁸ In the attempt to establish whether mitochondrial dysfunction could account for such an alteration, we analyzed mitochondrial structure and activity in situ in the same animal model (Ksp-Cre;Pkd1flox/− mice),³⁻⁸ bearing inactivation of Pkd1 in the distal tubules and collecting ducts of the kidney.¹⁵⁻¹⁹ Ksp-Cre;Pkd1flox/− mice display an early onset, severe cystic phenotype with already enlarged kidneys at postnatal day (P) P4 with the presence of numerous large cysts (Figure 1A) as compared to controls (Ksp-Cre;Pkd1flox/+). We analyzed mitochondrial mass and structure by imaging epithelial cells in kidney tubules from Ksp-Cre;Pkd1flox/− and control kidneys by transmission electron microscopy (TEM) (Figure 1) collected at P4, the same time-point in which we described the metabolic alterations.³⁻⁸ The number of mitochondria was quantified per area and their gross morphology was assessed separately in the renal cortex and in the medulla. To this end, serial sections of resin-embedded blocks of kidney were prepared, in order to have both kidney regions represented in each section for TEM imaging (Figure S1). Being the Ksp-Cre active in the distal portion of the nephron, the expression of Pkd1 in the proximal segment of the tubule in Ksp-Cre;Pkd1flox/− mice is expected to be unaffected, in line with the fact that no cysts are observed in this segment in Ksp-Cre;Pkd1flox/− mice. We, therefore, reasoned that this portion of the nephron might serve as an additional internal control.

![FIGURE 1](image)

Mitochondria alterations in cyst epithelial cells from Ksp-Cre;Pkd1flox/− kidneys. A, Representative images of kidneys from control and Ksp-Cre;Pkd1flox/− mice at P4. Scale bar, 1 mm. B, Representative TEM images of epithelial cells lining the cysts in kidneys from Ksp-Cre;Pkd1flox/− mice or in normal epithelia of controls in the indicated kidney regions from P4 mice. Scale bar, 1 μm. The area outlined in black is shown at higher magnification (right). C, Quantification of the number of healthy and damaged mitochondria in the indicated kidney areas of Ksp-Cre;Pkd1flox/− (KO) mice as compared to controls as assessed by TEM images. Data represent mean ± SD, one-way ANOVA, *P < .05, **P < .01
Indeed, we detected a reduction in the number of mitochondria in the cells of the cystic epithelia in Ksp-Cre;Pkd1floX−/− kidneys, either in the cystic cortex or in the cystic medulla (Figure 1B,C). Mitochondrial mass, on the contrary, is preserved in the proximal tubules of Ksp-Cre;Pkd1floX−/− kidneys (KO, cortex) as expected (Figure 1C and S1B). In the cystic epithelia, we observed swollen mitochondria as previously reported either in situ or on isolated cells derived from Pkd1 mutant mice, with few cristae and decreased electron density within the matrix, both in the cortex and in the medulla. Of interest, we also detected healthy, and normally appearing mitochondria scattered among damaged ones in the cysts suggesting that the absence of PC-1 does not per se result in this structural damage. We conclude that cells of the cystic epithelium have a reduction in mitochondrial mass and structural alterations as compared to control epithelial cells, but the alteration does not affect the totality of mitochondria suggesting that some residual mitochondrial activity might be present in the cystic epithelia.

3.2 | Drastic reduction in mitochondrial respiratory chain complexes in the cystic epithelium of Pkd1 mutant mice

We next aimed at testing whether mitochondrial respiration was affected in the cyst-lining epithelia of Ksp-Cre;Pkd1floX−/− mice in situ, and/or if some residual activity could instead be detected. To this end, we adapted to the mouse kidney the classical method used to measure the activity of electron chain complexes on the muscle biopsies of patients affected by mitochondrial disorders. We set-up and performed in situ enzymatic activity assays for SDH and COX, two of the five mitochondrial respiratory chain complexes (RCCs) on fresh-frozen kidney sections derived from wild-type or Ksp-Cre;Pkd1floX−/− mice at P4. To optimize the set-up of this technique to the kidney, we first used serial sections, in which staining with LTL and DBA was used to identify proximal and distal nephron segments, respectively (Figure 2A). We applied the technique to wild-type kidneys and detected the expected strong positive staining for both COX and SDH in the proximal tubules, in line with the known intense mitochondrial activity and abundance described in this segment (Figure 2B). We also found clearly detectable enzyme activities for both enzymes in the DBA-positive tubules indicating a measurable activity in distal tubules and collecting ducts in the normal kidney (Figure 2B). When a similar staining was applied to the kidneys derived from Ksp-Cre;Pkd1floX−/− mice, we observed a strong reduction of both COX and SDH activity staining in DBA-positive cystic epithelia of Ksp-Cre;Pkd1floX−/− mice as compared to control littermates (Figure 2C). This reduction in staining cannot be the consequence of the flattening of the cyst-lining cells compared to control tubule cells, because we could observe at high magnification some small cysts, DBA-positive with a cuboidal epithelium, that yet showed a very poor RCC activity staining (Figure 2D). In contrast, LTL-positive tubules in the kidneys derived from Ksp-Cre;Pkd1floX−/− mice did not show any reduction in RCC activity, in line with the lack of Cre recombinase activity in the proximal tubules and providing a good internal control for the staining (Figure 2A). These experimental findings indicate that inactivation of polycystin-1 results in a drastic decrease in mitochondrial respiration in the cystic epithelium in vivo.

3.3 | Inactivation of Pkd1 results in fragmentation of the mitochondrial network in cells of the renal cysts

Mitochondria are dynamic organelles that undergo cycles of fusion and fission events, and these events regulate the maintenance of a healthy organelle network. Tubular mitochondria participate to the network activity, while round-shaped organelles are in the fragmented state and are spared from the network. Recent studies have reported increased fragmentation of mitochondria in renal cells isolated from Pkd1 mutant mice. To assess whether fragmentation could be observed also in the Ksp-Cre;Pkd1floX−/− kidneys in vivo, we analyzed the TEM images acquired at P4 (Figure 1). Indeed, both morphologic and morphometric analysis of mitochondria on TEM images from Ksp-Cre;Pkd1floX−/− and control kidneys collected at P4 revealed profound alterations in the mitochondrial network at several levels (Figures 3 and S2). First, we detected a reduction in the average size of mitochondria in the epithelial cells in the cortex; in the medulla, where mitochondria of epithelial cells of controls are smaller than those in the control cortex, we observed a mild increase in the average area of mitochondria, possibly the result of mitochondrial swelling (Figure S2A-B). Second, a remarkable and significant increase in the number of round-shaped mitochondria in the cystic epithelia of the Ksp-Cre;Pkd1floX−/− kidneys as compared to controls could be appreciated (Figures 3B-D and S2C-D). These results indicate that in the cystic epithelia of Pkd1 mutant mice, indeed, the mitochondrial network is fragmented, with a large portion of mitochondria spared from the mitochondrial network.

3.4 | The mitochondrial fragmentation in cyst epithelial cells is possibly mediated by DRP1

Our studies indicate the presence of a mitochondrial network fragmentation. This event, however, can be the outcome of two different processes: decreased mitochondrial
FIGURE 2  Reduction of COX and SDH enzyme activities in Ksp-Cre;Pkd1^{floxed} kidneys. A, Representative images of COX and SDH enzymatic activity staining on total kidney serial sections of Ksp-Cre;Pkd1^{floxed} mice and control littermates at P4. LTL and DBA staining on adjacent serial sections were used to identify proximal and distal tubules, respectively. Scale bar, 1 mm. B, Representative images of COX enzymatic activity staining of kidney sections of control and Ksp-Cre;Pkd1^{floxed} mice at P4. The areas of DBA-positive tubules are highlighted. Scale bar, 100 μm. C, Representative images of COX and SDH enzymatic activity staining of kidney sections from Ksp-Cre;Pkd1^{floxed} mice and control littermates, at P4. Scale bar, 200 μm. D, Representative images of COX and SDH enzymatic activity staining on early cystic DBA-positive epithelium, highlighting the lack of enzymatic activity in the cuboidal epithelium (arrow). Scale bar, 50 μm.
fusion or increased mitochondrial fission. The molecular players that participate to these processes are well known: mitofusin (MFN) 1 and 2 drive fusion of the outer mitochondrial membrane (OMM) and Optic Atrophy 1 (OPA1) of the inner mitochondrial membrane (IMM); the cytoplasmic dynamin-related protein 1 (DRP1) is the master regulator of the mitochondrial fission and it translocates from the cytoplasm to the mitochondrial surface to induce mitochondrial division, upon post-translational modifications (Figure 4A).

We analyzed the expression levels of each of these proteins on total extracts from Ksp-Cre;Pkd1flox/− and control kidneys at P4, P8, and P10. The highly abundant, voltage-dependent anion-selective channel 1 protein (VDAC-1) of the OMM was used as a mitochondria loading marker. We detected an increase in the pro-fission protein DRP1 in Ksp-Cre;Pkd1flox/− kidneys at all-time points in comparison to control kidneys. This upregulation of DRP1 was evidenced by a significant increase in its mRNA expression levels in Ksp-Cre;Pkd1flox/− kidneys compared to control kidneys at all-time points (Figure 4A).
Expression of mitochondrial fusion and fission proteins is altered in Ksp-Cre;Pkd1^floox^- kidneys. A, Schematic representation of the molecular regulators of mitochondrial fusion and fission: pro-fusion proteins MFN1 on the OMM and OPA1 on the IMM, pro-fission protein DRP1, which is recruited from the cytosol to the mitochondria. B and C, Representative western blots for DRP1, MFN1, and OPA1 on total protein extracts from kidneys of Ksp-Cre;Pkd1^floox^- mice (KO) and controls (Ctrl) at P4, P8, and P10. VINC and VDAC1 are used as loading controls for total and mitochondrial proteins, respectively. D-F, Densitometric analyses of western blots in B and C. Data represent mean ± SD of three independent experiments, t test, *P < .05, ***P < .001. G, Immunofluorescence on kidney sections with TOM20 as mitochondrial marker. DAPI and DBA are used to identify the nucleus and the distal tubules, respectively. Scale bar, 10 μm.
control kidneys (Figure 4B,E). The levels of the pro-fusion proteins OPA1 and MFN1 in cystic kidneys are comparable to those detected in control kidneys at P4, while they are decreased at P8 and P10 (Figure 4C,F). Notably, western blot analysis confirmed a decrease in mitochondrial mass in Ksp-Cre;Pkd1flox/− kidneys at P10 but not at P4 (Figure 4B,D).

We reasoned that at P4 the number of mutant/cystic cells is still very low and downregulation of expression could not be appreciated because there is a dilution of the cystic contribution on total kidney extracts. Indeed, staining with TOM20 in DBA-positive cystic epithelia showed an appreciable reduction of mitochondrial mass also at P4 in comparison to control tubules (Figure 4G), in line with the TEM analysis (Figure 1C). Thus, our data indicate a downregulation of pro-fusion proteins OPA1 and MFN1 and a reduction in mitochondrial mass. Of interest, we did not observe any decrease in the expression levels of MFN2 (Figure S3), a molecule that is relevant not only for mitochondrial fusion, but also for tethering of the ER membrane to mitochondria, being it localized also on the ER side.13 These data further highlight that the increase in DRP1 is not secondary to mitochondria alterations and it is very likely to contribute and sustain the increase in mitochondrial fragmentation.

3.5 Pharmacological inhibition of mitochondrial fission by targeting DRP1 protein ameliorates PKD progression

Based on the studies above, we wondered whether decreasing mitochondrial fragmentation could be sufficient to ameliorate the cystic phenotype of Ksp-Cre;Pkd1flox/− mice. To this end, we used a recently reported DRP1 inhibitor, the mitochondrial division inhibitor Mdivi-1 23,24 (Figure 5A). To test for potential compound toxicity, control mice were treated for three days with IP injection of vehicle or 10, 25, or 50 mg/kg of Mdivi-1. Mild toxicity, measured as loss of body weight, was only detected with the highest dose (Figure 5B). Next, Ksp-Cre;Pkd1flox/− and control mice (Ksp-Cre;Pkd1floxed/− or Pkd1floxed/+, interchangeably) were treated with vehicle or the 25mg/kg daily of Mdivi-1 to assess whether this would achieve an improvement of the disease. To test whether the concentrations used are able to inhibit complex I as well, we performed OCR analysis on mitochondria isolated from control and cystic mice treated with 25 mg/kg of Mdivi-1 (Control mice, vehicle, N = 4, Mdivi-1, N = 6; cystic mice, vehicle, N = 6, Mdivi-1, N = 4) (Figure 5C). Mitochondrial respiration and respiratory control ratio were comparable between vehicle and Mdivi-1 treated mice, in either control or cystic mice (Figure 6D-F). Of note, incubation of isolated mitochondria with Mdivi-1 immediately after the OCR measurements reduced basal mitochondrial respiration when exposed to 50 or 100 µM concentrations in line with studies performed in cells, but not when exposed to nanomolar concentrations (50 or 100). Since the maximum plasmatic Mdivi-1 concentration after 20 mg/kg Mdivi-1 injection in mice was reported to be 46 nM, we conclude that this dosage is not sufficient to achieve inhibition of complex I in vivo.25 Furthermore, in line with recent findings by Manczak et al,24 we also observed increased OCR in kidney epithelial cells, mCCD cells incubated over night with 25 µM Mdivi-1 likely due to its effect on mitochondrial elongation, but reduced OCR after incubation with 75 µM Mdivi-1 possibly due to the effect on complex I as previously reported (Figures 6H and S5). Thus, our data demonstrate that the side effect of Mdivi-1 on complex I inhibition are only detectable at high concentrations and that the dosage used in vivo is not sufficient to achieve this effect on the mitochondria, at least in the kidney.
Based on our experimental findings, we conclude that decreasing mitochondrial fragmentation in vivo by inhibition of DRP1 protein ameliorates PKD progression. 

4 | DISCUSSION

In this study, we show that in cystic kidneys due to Pkd1 inactivation, epithelial cells of the distal tubules and collecting ducts have reduced mitochondrial mass and decreased activity of mitochondria RCCs in vivo. Mitochondria also appear swollen and damaged as previously reported in human tissues. Of interest, we also found healthy mitochondria scattered among aberrant ones, suggesting that the lack of Pkd1 is not per se sufficient to cause mitochondrial damage and that perhaps a progressive degeneration of mitochondria occurs in these tissues. Importantly, the mitochondrial network is fragmented because most mitochondria in vivo...
FIGURE 6  Treatment with Mdivi-1 decreased mitochondrial fragmentation without affecting mitochondrial respiration in Ksp-Cre;Pkd1flox/− kidneys. A, Representative TEM images of epithelial cells from kidney sections of Ksp-Cre;Pkd1flox/− mice at P8 treated with vehicle or Mdivi-1. B, Frequency distribution of individual mitochondria roundness in the indicated kidney regions of control mice (N = 2, Ksp-Cre;Pkd1flox/+ or Pkd1flox/+, interchangeably) and Ksp-Cre;Pkd1flox/− mice (cystic, N = 2) as assessed by TEM images. 1.0 = round-shape. Kolmogorov-Smirnov test, *P < .05, ****P < .0001. C, Experimental strategy to assess the effect of Mdivi-1 treatment on mitochondria respiration in kidneys. ETC, electron transport chain. D,E, Left graphs, representative analysis of OCR measurements of mitochondria isolated from the indicated mice in basal conditions and after sequential addition of ADP, oligomycin (O), FCCP, and antimycin A (AA) and corresponding respiratory states (2,3,4o,3u). Data are mean ± SD of at least four technical replicates; right graphs, analyses of respiratory states of mitochondria isolated from mice treated with vehicle of Mdivi-1. Control mice: vehicle, N = 4, Mdivi-1, N = 6; cystic mice, vehicle, N = 6, Mdivi-1, N = 4. Data are mean ± SEM. Unpaired t test, not significant (n.s.). F, Respiratory control ratio of OCR measurements in D and E. G, Basal OCR of isolated mitochondria after incubation with the indicated concentrations of Mdivi-1. Unpaired t test, ****P < .0001. H, Representative analysis of OCR measurements of mCCD cells in basal condition and after sequential addition of oligomycin A (O), FCCP, and antimycin A (AA), after over-night treatment with Mdivi-1 as indicated.
appear with a round-shape morphology. Consistent with this mitochondrial fragmentation, DRP1 protein levels are increased in *Ksp-Cre;Pkd1flox/−* kidneys and the treatment with the DRP1-inhibitor Mdivi-1 delays the progression of the disease. These data show that mitochondria are affected in *Pkd1* mutant cystic kidneys and that, irrespective of whether or not they are involved in driving cyst initiation, they do modify the progression of the disease.

Decreased mitochondrial mass and alteration in mitochondrial structure in cystic cells isolated from ADPKD mouse models have been reported. Of note, we also show a decrease in the activity of mitochondrial RCCs in the cystic epithelia of *Ksp-Cre;Pkd1flox/−* kidneys. COX and SDH in situ activity staining is a straightforward approach for investigating mitochondrial dysfunction, and it has been widely used to detect mitochondrial defects especially in skeletal muscle biopsies from patients with mitochondrial myopathies. The catalytic subunits of COX are encoded by the mitochondrial DNA, those of SDH are instead entirely encoded by nuclear DNA, and so its activity is not altered by impaired mtDNA. We detected the decrease in the activity of both RCCs, demonstrating a general defect in mitochondrial respiration, not strictly related to decreased mtDNA, in the cysts from PKD kidneys.

We showed that mitochondria in the cysts are decreased in number, and most of them are round-shaped as compared to the ones in the healthy tubules. Mitochondrial fusion and fission balance has now well been established to regulate mitochondrial metabolism and to participate to the metabolic cell state. In general, studies have shown that oxidative phosphorylation increases with fusion and decreases with fission to match the energy demand of the cells. Others and us reported changes in energetic metabolism in PKD cells and mouse models, and the mitochondrial fragmentation can in principle sustain this rewiring by lowering the efficiency of mitochondrial respiration and mitochondrial metabolism. It is important to note that previous work has shown that PC1 can also localize on the MAMs and lack of PC1 results in decreased mitochondrial respiration due to reduced mitochondrial Ca²⁺ uptake. Since Ca²⁺ entry in the mitochondria regulates matrix enzymes upstream or within the Krebs cycle, the observed increase in mitochondrial fragmentation could either contribute to the alteration in Ca²⁺ signaling between the ER and the mitochondria in the absence of PC1, or be secondary to such alteration. A similar reasoning can be applied to the recently reported role of the C-tail of PC1, which translocates into the matrix of mitochondria. The function for this protein domain in mitochondria has not been yet clarified, but the lack of its activity could in principle explain our current findings and the bioenergetics rewiring.

We assayed the expression levels of the known key molecular players of the fusion and fission machinery at different time points in the *Ksp-Cre;Pkd1flox/−* kidneys. We detected a higher amount of DRP1 protein in the cystic kidneys in comparison to control kidneys, and a concomitant down-regulation of OPA1 and MFN1. Importantly, mitochondrial mass was also decreased in our studies. DRP1 is a large cytosolic GTPase that is recruited to the outer mitochondrial membrane by different receptors. The best-known regulatory mechanism for mitochondrial fission involves phosphorylation of DRP1 by different kinases, many of them linked to signaling pathways activated by metabolic events. Phosphorylation can activate and stabilize the protein that drives mitochondrial fragmentation. We inhibited DRP1 with the specific inhibitor Mdivi-1, shown to attenuate mammalian mitochondrial division by targeting DRP1 self-assembly. *Ksp-Cre;Pkd1flox/−* mice treated with Mdivi-1 show an amelioration of the phenotype assessed as kidney enlargement and renal function. This indicates that mitochondrial fragmentation likely plays a role in cyst growth. Future studies should focus on studying whether or not mitochondrial dysfunction is sufficient to drive cystogenesis in the kidney. Our current data clearly show that mitochondrial network morphology regulation plays an important role in disease progression in the mouse, possibly modifying the phenotype.

We would like to stress here that our experimental findings also provide strong evidence on what is the effect of the inhibitor Mdivi-1 in vivo, at least in the kidney. Indeed, in line with previous work, we found that at the doses employed for treatment in vivo (20-25 mg/kg in the mouse) the effect appears to be exclusively on the inhibition of mitochondrial fragmentation, rather on inhibition of mitochondrial complex I. These data are also in line with the fact that Mdivi-1 had a protective effect on ischemia reperfusion injury, specifically in the kidney.

Improving mitochondrial metabolic activity has been shown to be an efficient strategy to ameliorate PKD phenotype. The miR-17 family modulates mitochondrial metabolism along with other pathways. Deletion of miR-17 – 92 results in improved expression of mitochondrial and metabolism-related genes and it attenuates cyst growth in two distinct PKD models. In line with this, treatment of PKD mouse models with Fenofibrate, a clinically available PPARalpha agonist, increased PPARalpha expression and attenuated cyst growth. In addition, The RhoA–YAP–c-Myc signaling axis has also been shown to promote PKD, and inhibition of RhoA signaling pathway by ROCK inhibitor Y-27632 or Fasudil ameliorates the phenotype in ADPKD mouse models. Of note, ROCK is one of the protein kinase that can phosphorylate DRP1 at amino acid S616 to promote fission. These pieces of evidence indicate that improving mitochondrial fitness can be achieved in different manners either on optimizing the metabolic function of mitochondria or by promoting mitochondrial fusion (current work), and it represents a novel therapeutic opportunity.
One final consideration goes to the potential role of mitochondrial dysfunction in ADPKD. While all the data reported to date in cellular and animal models carrying Pkd1 mutations provide a unified evidence of reduced mitochondrial calcium uptake, reduced mitochondria biogenesis, and reduced mitochondrial function, one recent study seems to suggest that the opposite occurs in Pkd2 mutants. Indeed, increased MAM contacts, increased calcium uptake, increased mitochondrial activity (oxygen consumption rate) were all reported in Pkd2 cellular and animal models, all effects mediated by an increase in MFN2 expression.\textsuperscript{13} Here, we show that on the contrary, Pkd1 mutants do not have increased MFN2 levels, in line with a previous study showing decreased Ca\textsuperscript{2+} uptake in the mitochondria.\textsuperscript{9} Despite these profound functional differences between Pkd1 and Pkd2 mutants, increased fragmentation was also observed in this last report. The generally accepted model is that disease-relevant activities are very similar between PC1 and PC2, because loss-of-function mutations in either PKD1 or PKD2 in patients result in a very similar phenotype. So, perhaps the shared feature between these two models, that is, increased mitochondrial fragmentation, should deserve careful attention for future studies. Our current data suggest that inhibiting the mitochondrial-fragmenting machinery improves disease progression, at least in Pkd1 mutant mice. It should be noted that the role of Mdivi-1 as a specific inhibitor of DRP1 has been controversial with some investigators reporting that high doses of Mdivi-1 can achieve inhibition of complex I in mitochondria.\textsuperscript{34} In our studies, we have shown that the usage of 25 mg/kg of Mdivi-1 achieves mitochondria elongation in the kidney, in line with its role as an inhibitor of DRP1. Therefore, our data strongly suggest that achieving mitochondrial elongation in the cystic epithelia results in improved disease progression. However, future work should focus on performing genetic interaction studies to manipulate key molecules governing the process of fusion and fission (such as OPA1, MFN1, and DRP1) in the renal epithelia to establish in a more robust manner their involvement in disease initiation and/or progression.

In conclusion, our studies demonstrate that DRP1-driven mitochondrial fragmentation is a hallmark of the cystic epithelium and its inhibition retards disease progression in PKD. Future work is warranted to elucidate the molecular mechanisms driving mitochondrial fission and the role of PC1 (and/or 2) in these cascades of events.

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**CONFLICT OF INTEREST**

AB and MC are coinventors on a European patent application N. 13733319.1 and US Patent application N. 14/413,280 related to glycolysis inhibition for the treatment of PKD; AB is coinventor on a European application (undisclosed number-under secrecy) related to the modulation of other metabolic pathways for the treatment of PKD.

**AUTHOR CONTRIBUTIONS**

L. Cassina designed research, performed research, analyzed data, and wrote the paper; M. Chiaravalli designed research, performed research, analyzed data; A. Boletta designed research, analyzed data, and wrote the paper.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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