Preclinical validation and mechanistic understanding of drug repurposing candidates for polycystic kidney disease

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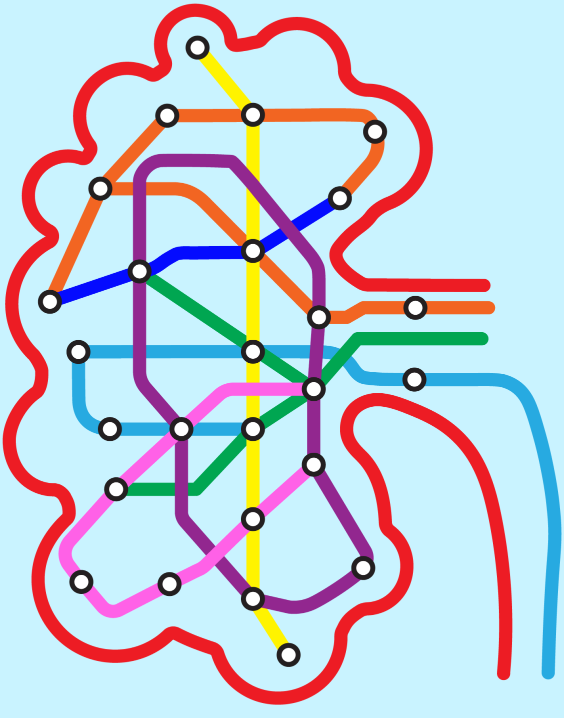
CHAPTER 1

General introduction



Kidney & cysts

The kidney is one of the key organs in the body, responsible for the removal of waste products and toxins from the blood stream, as well as controlling body fluid levels and electrolyte concentrations. The functional element in the kidney is known as a nephron, of which 1 million are present in the average adult kidney. Nephrons can be split into two structural parts, the glomerulus, in which the blood is filtered, and the tubule, in which nutrient reabsorption takes place until eventually urine is produced and transported via collecting ducts to the bladder. About 200 litres of blood are filtered every day by nephrons, resulting in the production of 1-2 litres of urine. In each tubule, essentially a system of convoluted tube-like structures, four major sections can be distinguished: the proximal tubule, the loop of Henle, the distal tubule and the collecting duct. Each section has a specific function in nutrient reabsorption and therefore each section can be characterized by its own epithelial cells, each with their own set of genes and proteins that are expressed.

While tubular epithelial cell proliferation is very low during homeostasis in the adult kidney, this increases for repair of the epithelium, most commonly in response to toxin exposure, injury and/or blockages of the tubule. However, in a small percentage of repair events, this is done incorrectly, resulting in epithelial cells budding off from the tubular wall and eventually detaching, forming a fluid-filled balloon-like structure known as a cyst. This incidental cyst formation is common in the general population, normally unrelated to disease and harmless. This is in contrast to cyst formation caused by various genetic mutations, which result in the formation of cysts that grow progressively in size and number in the kidney, until renal failure is reached (Figure 1). Diseases characterized by this type of cyst formation are grouped together under 'polycystic kidney disease', first reported in the autopsy report of Polish king Stefan Bathory, whose kidneys were described as 'large like those of a bull, with an uneven and bumpy surface'[1]. A large number of conditions are classified under polycystic kidney disease, but the most common and the most studied of these is autosomal dominant polycystic kidney disease (ADPKD).

ADPKD

As the most common type of cystic kidney diseases, ADPKD affects between 1:400 to 1:2500 people worldwide, meaning that over 10 million people worldwide are predicted to be affected by the disease[2]. A mutation in either the *PKD1* (± 80%) and *PKD2* (± 15%) gene is the main cause of the disease, with the remaining cases most commonly being caused by mutations in other genes, such as *GANAB*, *DNAJB11*, *IFT140*, *ALG8* and *ALG9*[3-7]. In early life, ADPKD patients are normally asymptomatic, but from the second decade of life, cysts are large enough to be detected and will increase in size and number over time. This lowers kidney tissue function and is commonly accompanied by hypertension, proteinuria (excessive protein levels in the urine), haematuria (blood in the urine), abdominal pain, nephrolithiasis

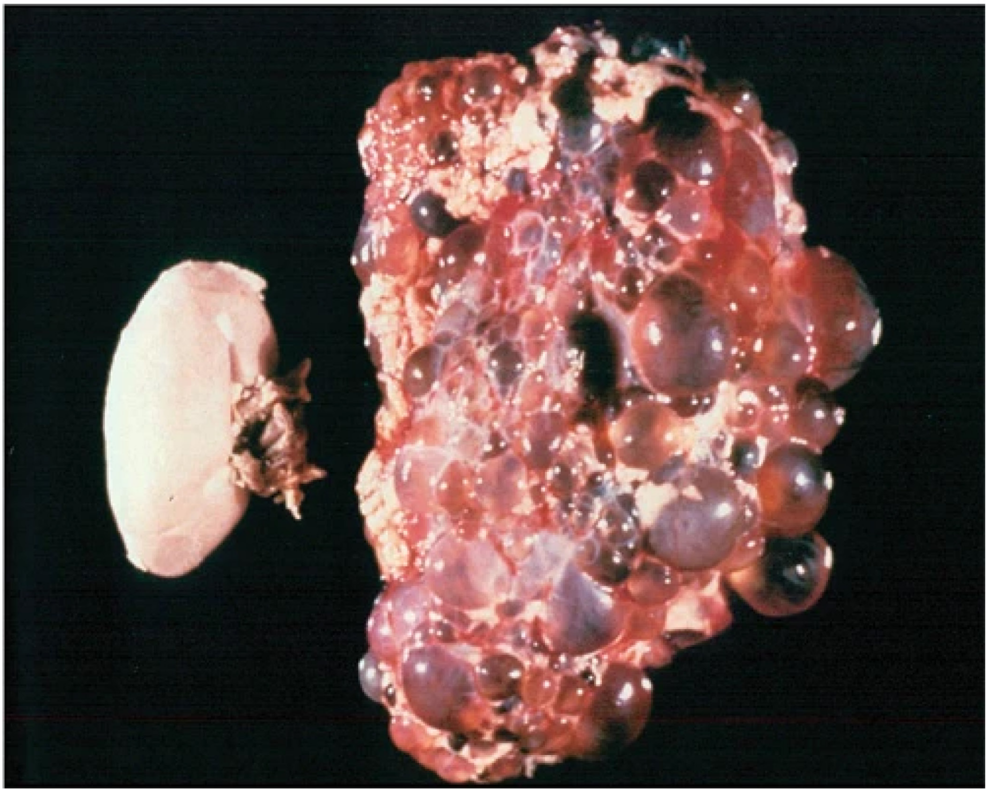


Figure 1: Comparison of a normal human kidney (left) with an end-stage human autosomal dominant polycystic kidney (right).
The cystic kidney is clearly increased in size and has grown large fluid-filled cysts. Image from Calvet JP, Nature Genetics 2003[275].

(kidney stones), urinary tract infections, and problems with urine concentration. In addition, cysts may develop in other organs, with the liver, pancreas and seminal vesicles most commonly affected. Other extrarenal manifestations include intracranial aneurysms, cardiac valve irregularities and cardiomyopathy[2,8]. ADPKD is progressive in nature, meaning that around 50-60 years of age, patients reach end-stage renal disease (ESRD, or renal failure), needing renal replacement therapy (dialysis or kidney transplantation). Diagnosis options include abdominal imaging using ultrasound, CT and/or MRI or by screening for *PKD1* and/or *PKD2* mutations via gene-panel analysis or whole-exome sequencing[9-11]. Difficulties arise with genetic screening due to the large size of the *PKD1* gene, the number of *PKD1* pseudogenes in the human genome (6) with a highly similar genetic sequence and the high level of allelic heterogeneity in both PKD1 and PKD2, but this is lessened by technological developments, however abdominal imaging is still the first option[11-17].

Current treatment options are limited, as only one drug is approved for patient use thus far, the vasopressin V2 receptor (V2R) antagonist tolvaptan (Jinarc®). Tolvaptan has received regulatory approval in recent years in the Japan, Canada, the European Union, Switzerland, South Korea and the United States. By acting on V2R, tolvaptan lowers intracellular cyclic AMP (cAMP) levels, thereby slowing cyst growth. Clinical trials showed that tolvaptan can slow the rate of kidney growth, as well as estimated glomerular filtration rate (eGFR) decline in both early- and late-stage ADPKD patients[18-20]. However, tolvaptan is associated with multiple adverse effects, most commonly polyuria and hepatotoxicity, which means that the drug is only available to a subset of patients, those with rapidly progressive ADPKD[21]. Other treatment options remain elusive up till now, and the current treatment paradigm consists of monitoring and managing kidney function, cyst progression, blood pressure and pain[2].

The polycystin proteins

The human *PKD1* gene is located on chromosome 16 (16p13.3), contains 46 exons and is widely expressed, with transcripts detected in virtually all tissues[22,23]. The level of expression differs throughout life, with higher levels detected during embryonic development and lower levels in adulthood[23,24]. *PKD1* encodes the protein polycystin-1 (PC-1), a transmembrane protein of 450 kDa and containing 4303 amino acids[22]. The protein has a large extracellular N-terminal segment, 11 transmembrane domains and a small intracellular C-terminal tail (Figure 2)[25]. As such, its structure is reminiscent of a G-protein coupled receptor (GPCR), and indeed, PC-1 is increasingly being described as an adhesion GPCR. Various studies have already shown that PC-1 interacts with G proteins via their Gα subunits through its C-terminal tail and can regulate signalling activity downstream of these G proteins[26-33] In addition, manipulation of the PC-1-G protein interactions affects the development of a cystic phenotype in *Xenopus* embryos and mice[34,35]. Like other adhesion GPCR families, PC-1 contains multiple domains that are involved in protein-carbohydrate, protein-protein and protein-matrix interactions, of which the GPCR-Autoproteolysis Inducing (GAIN) domain is the most promiment[36-38]. In addition, both PC-1 and other adhesion GPCRs contain a GPCR proteolytic site (GPS) in their GAIN domains, where autoproteolytic cleavage can take place[39]. PC-1 cleavage at the GPS results in an extracellular N-terminal fragment and an intracellular C-terminal fragment. The N-terminal fragment then undergoes conformational changes, revealing a 'stalk' peptide which can non-covalently bind to the remaining transmembrane region, inducing G protein signaling[40,41]. It has since been demonstrated that GPS cleavage is vital for proper PC-1 trafficking and function[39,42,43]. The C-terminal part, when cleaved from the full protein, can translocate to the nucleus, where it modulates Wnt or STAT signalling, or to the mitochondria, where it alters mitochondrial function and potentially energy metabolism[44-47]. The C-terminal part is also used to communicate with polycystin-2 (PC-2), the other polycystin protein encoded by *PKD2*[48,49]. *PKD2* can be found on chromosome 4 (4q22.1) and has 15 exons, the corresponding PC-2 protein (110 kDa) has 968 amino acids.

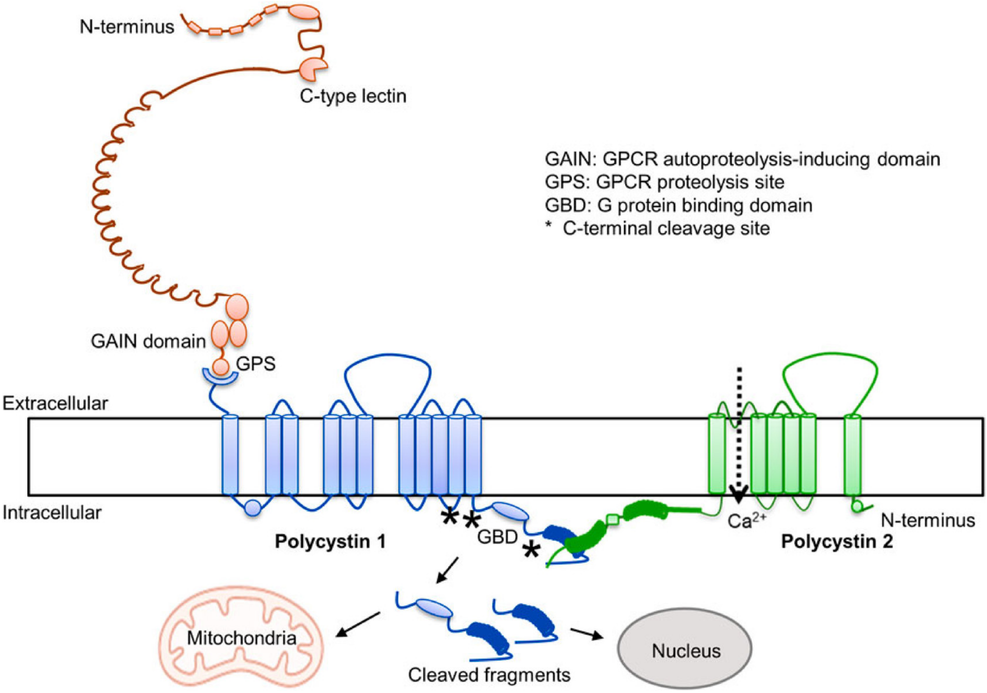


Figure 2: Schematic representation of the structure of the polycystin proteins and their intracellular cleaved fragments. Image from Zhou X & Torres VE, Front Mol Biosci 2022[276].

Structurally, PC-2 consists of six transmembrane domains and a C-terminal tail containing a calcium binding motif (Figure 2)[49]. PC-2 is a transient receptor potential family member and serves as a non-selective cation channel, conducting Ca^{2+}, K^+ and Na^+[48,49]. PC-1 and PC-2 can be found at different sites in the cell; PC-1 at the plasma membrane and lateral junctions, with PC-1 fragments translocating to the mitochondria and nucleus[41,50]. PC-2 is found at the basolateral membrane and the endoplasmic reticulum (ER)[50]. Both are also found in the primary cilium, an organelle responsible for converting environmental cues into cellular signalling to maintain tissue homeostasis[51,52]. PC-1 and PC-2 can form heterotetramers with 1:3 stoichiometry, functioning as ion channels, which a higher Ca^{2+} ion conductance than homotetramer PC-2 channels [25,53-56]. In fact, the ion channel function is directly affected by PC-1 through its contribution to the channel pore[55,57].

Cyst formation

Multiple models have been proposed to describe the initial events underlying cyst formation. ADPKD patients are born with a wildtype *PKD1*/*PKD2* allele and a germline mutation in the other allele. The *second hit* model proposes that throughout life, the wildtype allele will also be affected by a somatic mutation, lowering the level of functional PC-1/PC-2, and consequently, priming the renal epithelium for cyst formation[58-60]. This model is supported by individual cyst analyses that confirm point mutations or deletions in the wildtype allele. The second hit model has been expanded with findings that germline mutations in one PKD gene (i.e. *PKD1*) can be followed by somatic mutations in the other PKD gene (i.e. *PKD2*), so-called *transheterozygous* mutations[60,61]. The *gene dosage* model proposes that stochastic fluctuations in the PC-1 protein level might lower the level of functional protein below a critical threshold, priming the renal epithelium for cyst formation. Evidence supporting this model shows that both overexpression and lower expression of the PC-1 protein results in cyst formation[62-64]. No definitive evidence exists for either model at this point, and it is very possible that the models are not mutually exclusive, and occur virtually simultaneously in the cystic kidney[65]. Multiple studies have demonstrated that a third hit (after germline and somatic mutations), in the form of renal injury, is often required for cystogenesis, adding further complexity to the cystogenesis process[66-69].

Polycystin dosage falling below a critical threshold, either through gene mutation or stochastic fluctuation, is the primary step for cystogenesis, but the downstream mechanisms from that point remain to be identified. It has been proposed from studies with knock-out mice for *Pkd1* and cilia that a cilia-dependent cyst activation (CDCA) signal exists, as mice with a *Pkd1* and cilia KO, and mice with only a cilia KO, develop less cysts compared to *Pkd1* KO mice[70-72]. The Hedgehog pathway has been investigated as possible contributor to the CDCA signal, but was found not to be involved[73]. While more information has been revealed in recent years about the function of the PC-1/PC-2 complex within the cell, in particular in relation to its adhesion GPCR and ion channel functions, the exact mechanism as to how downstream signalling pathways are regulated remains to be further elucidated.

Dysregulated intracellular signalling

After formation of an initial cyst, both mechanical stress and injury-related mechanisms result in aberrations in various processes, such as proliferation and fluid transport, which promote formation and expansion of new cysts in a snowball-like fashion[74]. This culminates in the total dysregulation of the intracellular signalling machinery, with many pathways affected, that all contribute to the excessive cyst growth, and eventually, renal failure.

Proliferation/cAMP/calcium

The polycystin proteins and cAMP signalling are closely interlinked through calcium signalling. PC-2 is a non-selective cation channel, and can form heterotetramer complexes together with PC-1, with PC-1 regulating the gating of the ion channel complex. In addition, PC-1 can also regulate the calcium influx through the PC-2 homomeric calcium channels[55,57,75,76]. As such, intracellular calcium levels are dysregulated in ADPKD, which also affects intracellular cAMP levels through activation of adenylate cyclase (AC) 5 and/or 6[77,78]. In addition, phosphodiesterases (PDEs), responsible for cAMP breakdown, are less expressed in ADPKD tissue, resulting in increased intracellular cAMP levels[79]. cAMP is also affected through calcium-independent mechanisms. In the early stages of ADPKD, impaired urine concentrating results in elevated circulating vasopressin levels[80-83]. This results in V2R overactivation and subsequently, in AC6 overactivation (Figure 3).

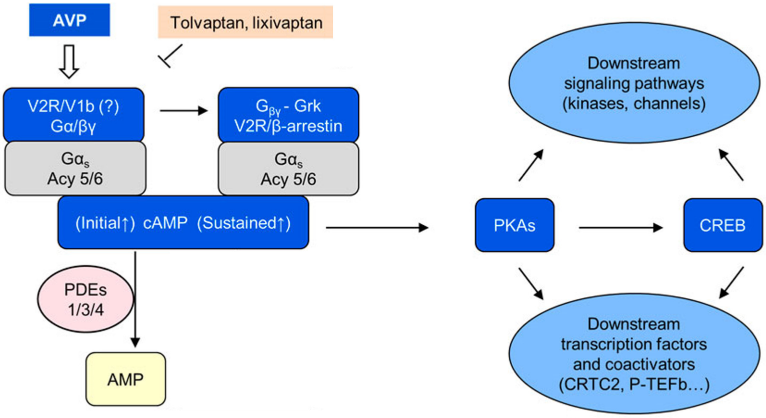


Figure 3: Overview of altered cyclic adenosine monophosphate (cAMP) signalling effects in ADPKD.

Due to binding of vasopressin (AVP) to the vasopressin V2 receptor (V2R) results via G-protein coupled receptors to the activation of adenylate cyclase 5/6 (Acy 5/6), the enzymes responsible for cAMP production. Increased cAMP in turn results in the activation of protein kinase A (PKA) and cAMP-responsive binding element (CREB), which are both responsible for the activation of downstream signalling pathways, transcription factors and co-activators. cAMP can be converted to AMP via phosphodiesterase (PDE) enzymes, which are less active in ADPKD. Inhibition of V2R with tolvaptan or lixivaptan inhibits the pro-cystic cAMP-mediated effects. Image adapted from Zhou X & Torres VE, Front Mol Biosci 2022[276].

The increased levels of intracellular cAMP have been well described in ADPKD models, as well as the mechanisms through which it promotes ADPKD disease progression[84-87]. cAMP increases epithelial cell proliferation in ADPKD through sequential phosphorylation of PKA, B-Raf, MEK and ERK, a process that can be inhibited by restoring normal calcium levels[88-90]. cAMP also promotes cystogenesis through increased fluid secretion driven by the exit of chloride ions via PKA-dependent phosphorylation of the apical cystic fibrosis transmembrane conductance regulator (CFTR)[91,92]. Together with the increased transport across the basolateral membrane of Na^+ and K^+ ions, this induces osmotic pressure, which forces fluid excretion into the cyst lumen.

In addition to promoting cyst growth, it has also been shown that cAMP might have a role in cyst formation. Increased cAMP levels can disrupt regular tubulogenesis by PKA-dependent enhancement of the Wnt/β-catenin signalling axis[93-95]. In addition, PC-1 deletion switches tubule formation to cyst formation in principle-like Madin-Darby canine kidney (MDCK) cells via increased cAMP levels, while in PC-1 containing cells, pharmacologically increased cAMP causes cyst formation and mis-orientation reminiscent of PC-1 deficient cells. This misorientation was also observed in metanephric kidneys with pharmacologically increased cAMP levels[96].

Metabolic reprogramming

Due to the excessive cell proliferation observed in ADPKD, as well as other biochemical similarities, ADPKD has in the past been referred to as a 'neoplasia in disguise'[97]. A prominent feature in tumour cells is the metabolic shift from oxidative phosphorylation to aerobic glycolysis, better known as the Warburg effect. This shift results in low adenosine triphosphate (ATP) generation, but allows tumour cells higher nutrient availability and building blocks to facilitate their excessive proliferation (Figure 4)[98-100]. This metabolic rewiring was also observed in mouse *Pkd1* and human *PKD1* mutant cells and tissues, and is accompanied with lactate production, increased expression of glycolytic enzymes, increased intracellular ATP, overactivation of mammalian target of rapamycin (mTOR) complex 1 (mTORC1), inhibition of adenosine monophosphate (AMP)-activated kinase (AMPK) and dysregulation of metabolic transcription factors[101-105]. Consistent with these findings are the observations that high glucose concentrations and hyperglycaemia promote cystogenesis and disease progression[106,107]. Although the Warburg effect is yet to be proven in ADPKD patients, its importance is further highlighted by the increased activity of the pentose phosphate pathway in *Pkd1*$^{-/-}$ cells[105]. This pathway branches off from glycolysis after the formation of glucose-6-phosphate, and is vital for the biosynthesis of ribonucleotides and fatty acids, which are both used in anabolic processes to facilitate the excessive proliferation observed in ADPKD[108].

As part of the metabolic shift from oxidative phosphorylation to aerobic glycolysis, *Pkd1*$^{-/-}$ cells transform pyruvate to lactate, instead of using it to fuel the tricarboxylic acid (TCA) cycle. However, to maintain a mitochondrial membrane potential (and prevent cell death), the TCA cycle must remain active. Therefore, *Pkd1*$^{-/-}$ cells require an alternative carbon source to do so. Perhaps unsurprisingly, *Pkd1*$^{-/-}$ cells utilize the same source as tumour cells, namely the amino acid glutamine, through glutamine anaplerosis. In this process, tumour cells convert glutamine to glutamate and then to α-ketoglutarate, a TCA cycle intermediate, which then fuels the TCA cycle. In addition, α-ketoglutarate also is used to produce citrate, which in turn forms a building block for lipids[109-111]. *Pkd1*$^{-/-}$ cells use the same mechanism for TCA fuelling, for example through glutaminase 1[112-114]. However, *Pkd1*$^{-/-}$ cells can also utilize other enzymes for this purpose, such as asparagine synthetase (conversion of aspartate to asparagine while deamidating glutamine to glutamate) or arginosuccinate synthase 1 (required for arginine synthesis; lower expression of the enzyme in *Pkd1*$^{-/-}$ cells and tissues increases glutamine utilization)[105,115].

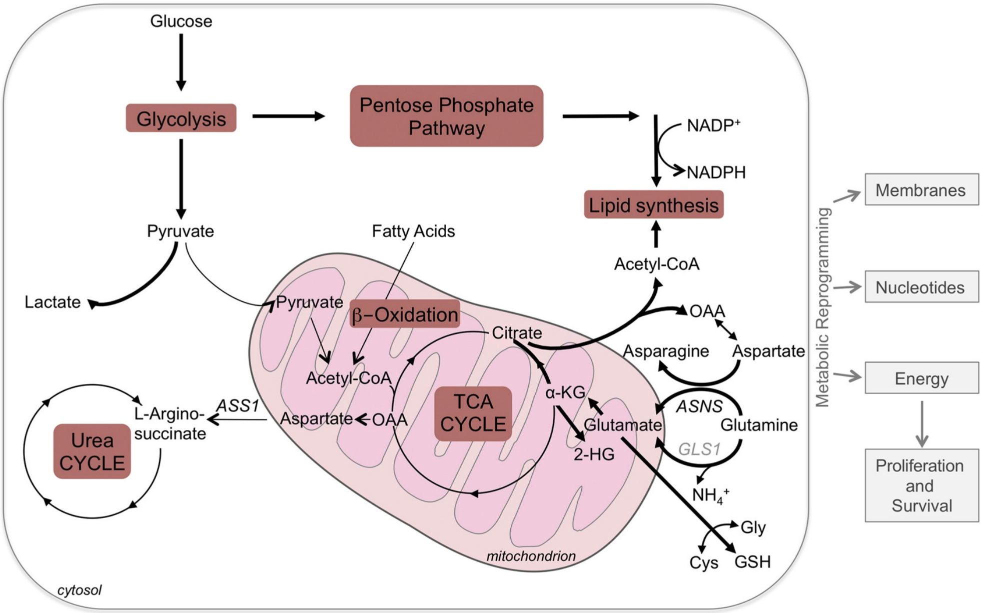


Figure 4: Overview of the reprogrammed metabolic pathways in ADPKD.

Pathways supportive of cell proliferation, such as glycolysis, pentose phosphate pathway are more active in *Pkd1*$^{-/-}$ cells and tissues, to the detriment of catabolic pathways such as β-oxidation, the TCA cycle and the urea cycle. The reprogramming of metabolic pathways results in an altered flux of metabolites throughout the different pathways, as indicated by the arrows (increased flux is indicated by thick arrows). 2-HG = 2-hydroxyglutarate, ASNS = asparagine synthetase, ASS1 = arginosuccinate synthase 1, α-KG = α-ketoglutarate, CoA = co-enzyme A, Cys = cysteine, GLS1 = glutaminase 1, Gly = glycine, GSH = glutathione, OAA = oxaloacetate, TCA = tricarboxylic acid. Image from Podrini C et al., Cell Signal 2020[277].

Other than carbohydrates and amino acids, lipids also are an important fuel source for the proliferating cell. As such, it is to be expected that lipid metabolism is also hijacked in ADPKD, and this is indeed the case, although the molecular mechanisms underlying this are less elucidated than their carbohydrate and amino acid counterparts. The main characteristic of the hijacked lipid metabolism in ADPKD is a reduction in fatty acid oxidation (FAO)[116,117]. Urinary acetylcarnitine, an important FAO regulator, has been shown to correlate with disease progression[118]. Also, reduced FAO is partly caused by the reduced expression of the two transcription factors peroxisome proliferator-activated receptor alpha (PPARα) and hepatocyte nuclear factor 4 alpha (HNF4α) in ADPKD[117,118]. Reduced PPARα activity is regulated by the oncogene c-Myc through microRNA-17 in both ADPKD and cancer, which further emphasizes the similarities between *Pkd1*$^{-/-}$ and cancer cell metabolism[119,120]. Moreover, the necessity for increased fatty acid synthesis (FAS) in rapidly proliferating cells (for example to facilitate membrane production for new cells) activates a feedback loop, inhibiting fatty acid transporters carnitine palmitoyltransferase 1 (CPT1) and CPT2 in ADPKD[105]. In this light, it is interesting to note that mutations in the *CPT2* gene can result in a severe case of infantile PKD[121].

The amount of metabolic alterations found in ADPKD also has consequences for mitochondrial function. The mitochondria are the powerhouse of the cell, and as such, are home to many metabolic pathways, including the TCA cycle and oxidative phosphorylation. Reduced oxidative phosphorylation is a hallmark of ADPKD, and has extensively been demonstrated in various murine models, as well as human ADPKD cysts and patient-derived cells, both functional (through measurement of ATP synthesis and the oxygen consumption rate) and transcriptional (reduced expression of genes involved in oxidative phosphorylation)[52,101,102,105,116-118,122,123]. The functional defects are accompanied by structural changes as well. Both mouse models and patient tissue have shown that ADPKD cells show swollen mitochondria with abnormal cristae that are less elongated, as well as reduced mitochondrial biogenesis due to reduced activity of PPARα and PPAR–gamma coactivator 1-alpha (PGC1α)[44,117,122,124]. Another observed defect is the increased fragmentation of mitochondria in *Pkd1*$^{-/-}$ and *Pkd2* knock-down cells, indicating mitochondrial dysfunction[124,125]. The major dysfunction of mitochondria in ADPKD is not surprising, as several studies have shown that the polycystin proteins can directly influence mitochondria. Both PC-1 and PC-2 can influence calcium signalling (as discussed before), in which the mitochondria also play an important role. An increased mitochondrial Ca^{2+} concentration increases mitochondrial gene expression, required to sustain and increase oxidative phosphorylation and ATP synthesis[126]. The source of mitochondrial calcium are the mitochondria-associated ER membranes, and studies have shown that both polycystin proteins can localize to these membranes[52]. Intriguingly, reduced PC-1 activity results in less mitochondrial influx, while reduces PC-2 activity results in the opposite[52,125]. PC-1 can also directly influence mitochondrial morphology and function, as the PC-1 C-terminal tail can be cleaved and translocated to the mitochondrial matrix[44].

Inflammation and immunity

Histological analysis of both early-stage and late-stage kidneys of ADPKD patients revealed apparent signs of inflammation in both stadia of the disease[50,127]. As such, interstitial inflammation and a large presence of cytokines and inflammatory cells are considered a hallmark of ADPKD. Analysis of both patient tissue and cyst fluid revealed the presence of several cytokines (or genes encoding them), among which monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor alpha (TNFα) and several interleukins are most prominent and the best described[128-130]. MCP-1, encoded by the *Ccl2* gene, is a ligand for the CCR2 receptor, which can be found on monocytes and T-cells[131,132]. Its presence is elevated in tissues, cyst fluid and urine from both rodent and human origin, and urinary MCP-1 levels inversely correlate with renal function[133-136]. The importance of MCP-1 in disease progression was further emphasized by a *Ccl2;Pkd1*$^{-/-}$ double knock-out mouse, which displayed reduced macrophage numbers, slower cyst growth and improved renal function, compared to a *Pkd1*$^{-/-}$ single knock-out mouse. Treatment with a CCR2 inhibitor showed similar effects[137]. Another key cytokine in ADPKD pathogenesis is TNFα, which also has been shown to be elevated in patient cyst fluid[128,138]. In collecting duct cells, TNFα can disrupt polycystin interactions and alter the subcellular localization of PC-2, and it also influences many other signalling pathways and processes known to be dysregulated in ADPKD, such as proliferation, apoptosis and inflammation[138-140].

The elevated cytokine production by the renal epithelium in ADPKD consequently results in the recruitment of immune cells, such as mast cells and neutrophils. Both have been shown to be present in human cystic kidneys, and neutrophils have also been detected in animal models[141-146]. However, the most prominent immune cell contributing to disease progression, and therefore the most researched, is the macrophage. Macrophages are part of the innate immune system, and are responsible for the phagocytosis of foreign materials, secreting both pro- and anti-inflammatory cytokines in the process, which eventually results in tissue repair. They can be classified based on origin, as either infiltrating (bone-marrow derived) or tissue-resident (embryonically derived)[147,148]. The presence of macrophages in cystic tissues, both human and animal, has been described extensively over multiple decades, which consequently was followed by several genomic studies revealing overexpression of innate immune response genes[101,133,149-153]. More recent studies revealed that macrophages also directly contribute to disease progression and are pro-proliferative, as F4/80$^+$ (mice) and CD163$^+$ (human) macrophages were accumulating in cyst-lining regions and the interstitium. Treatment with liposomal clodronate, which depletes macrophages, reduced various cystic disease parameters, as well as excessive epithelial proliferation[154,155]. Macrophage effects on cell proliferation were also confirmed by findings that (1) macrophage migratory inhibitory factor (MIF) is required for macrophage retention, and that both genetic deletion and pharmacological inhibition of MIF slow down cyst growth, and (2) arginase 1 (Arg1) expressing macrophages directly communicate with the cystic epithelium, stimulating proliferation, and that Arg1 inhibition reduces cyst growth and proliferation[135,156]. The exact contributions of both infiltrating and resident macrophages are difficult to separate, as both types secrete similar sets of cytokines. *Pkd1*$^{-/-}$ mice display cyst formation, increased MCP-1 expression, as well as an increased presence of CCR2$^+$ macrophages, however it is unknown if these macrophages are infiltrating or resident[157]. Both inhibition and genetic deletion of MCP-1 reduce the cystic burden, which suggests a role for infiltrating macrophages in disease progression, although this has recently been questioned[137,157,158]. The overlapping sets of cytokines secreted by infiltrating and resident macrophages are likely regulated by the transcription factor interferon regulatory factor 5 (IRF5), which is increased in both cell types[153].

While the role of innate immune cells, and specifically macrophages, has been researched extensively, much less is known about the role of the adaptive immune system. The presence of lymphocytes in both cystic tissue (human and mouse) and cyst fluid has been described extensively, which indicates they do play a role in disease progression[128,149,151,159,160]. Recent studies have found increased T-cell numbers in mouse and human kidneys, which correlate with disease severity[161,162]. Of note here is that immunodepletion of $CD8^+$ T-cells exacerbates disease progression in mice, which is associated with decreased apoptosis and increased proliferation of the epithelium[161]. This suggests that $CD8^+$ T-cells might have a protective role in ADPKD disease progression.

Other than the attraction of immune cells, the release of cytokines by the cystic epithelium also results in the overactivation of several pro-inflammatory signalling pathways, which reinforce the inflammatory reaction. The most relevant pathways in this regard are the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) pathway and the Janus kinase-signal transducer and activator of transcription proteins (JAK-STAT) pathway, which both were identified as upregulated in human cystic kidney tissue[101]. The NFkB pathway can be activated by a variety of cytokines, such as MCP-1, TNFα and interleukin 6, that bind to Toll-like receptors (TLRs)[163,164]. Most cytokines, in turn, are also upregulated by NFkB pathway activation, creating a positive feedback loop[165]. Several studies have shown that different pathway effectors of the NFkB pathway, such as p65, IKKα/β and NFkB, are increased in both mouse and human cystic tissues, and result in the increased expression of pro-inflammatory genes[166-168]. The JAK-STAT pathway is activated by a multitude of cytokines, such as interferons and interleukins, as well as multiple growth factors[169]. Pathway activation results in JAK activation, which in turn can phosphorylate different STAT transcription factors, such as STAT3 and STAT6. These then translocate to the nucleus and activate the transcription of pro-inflammatory and pro-fibrotic genes. Several studies have shown that the polycystin complex, the PC-1 C-terminal tail and membrane-bound PC-1 can interact with STAT1, STAT3 and STAT6, all three of which are overexpressed in both mouse and human cystic kidneys[46,101,170-173].

Therapeutic strategies & trials

Due to the diverse range of dysregulated signalling pathways in ADPKD, many possibilities exist for therapies interfering with this dysregulation. However, such a compound will only be a symptomatic treatment and not a curative one, as the underlying cause, mutations in the *PKD1* or *PKD2* genes, are not addressed. While multiple options for potential interventions are possible, it is important to consider the duration of a potential treatment. As ADPKD patients will suffer from the disease for their whole lifetime, a treatment has to be safe in this long period and without major side-effects, while maintaining its efficacy. As such, it has been difficult to identify, test and approve a new treatment that can meet all these criteria. Nevertheless, progress has been made in this regard (Figure 5).

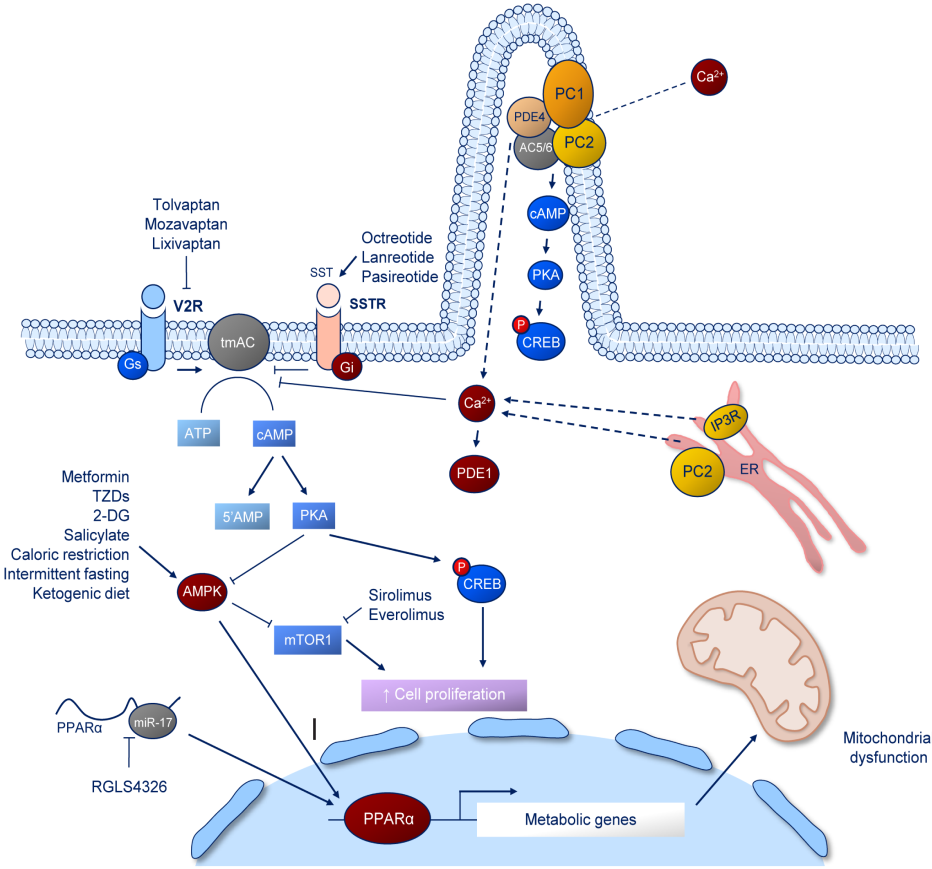


Figure 5: Overview of tested (pre)clinical drug candidates and associated pathways in autosomal dominant polycystic kidney disease (ADPKD).
2-DG = 2-deoxyglucose, AC5/6 = adenylyl cyclase 5/6, 5'AMP or AMP = adenosine monophosphate, AMPK = adenosine monophosphate-activated kinase, ATP = adenosine triphosphate, cAMP = cyclic adenosine monophosphate, CREB = cyclic adenosine monophosphate-responsive element, ER = endoplasmic reticulum, Gi = Gi protein alpha subunit, Gs = Gs alpha subunit, IP3R = inositol 1,4,5-triphosphate receptor, miR-17 = miR-17 microRNA precursor family, mTOR1 = mammalian target of rapamycin 1, PC = polycystin, PDE = phosphodiesterase , PKA = protein kinase A, PPARα = peroxisome proliferator-activated receptor alpha, SST = somatostatin, SSTR = somatostatin receptor, tmAC = transmembrane adenylyl cyclases, TZDs = thiazolidinediones, V2R = vasopressin V2 receptor. Adapted from Zhou JX & Torres VE, Adv Kidney Dis Health 2023[182].

Vasopressin V2 receptor antagonists

As described before, V2R has an important role in driving disease progression. Vasopressin levels are elevated in ADPKD patients, and as a consequence, the receptor is overactivated[80-83]. This then leads to AC6 overactivation and then elevated intracellular cAMP concentrations, which drive excessive cell proliferation and cyst progression, mainly in the collecting duct and distal tubule segments[77,78,88-90,174]. This prompted further preclinical research into V2R antagonists, of which multiple showed efficacy in cellular and animal models[85,174-177]. Due to these positive results, the TEMPO 3:4 phase 3 clinical trial was started in 2007 with tolvaptan, and showed that tolvaptan slows down the total kidney volume (TKV) increase and slows the rate of declining kidney function (measured by the estimated glomerular filtration rate, eGFR) in ADPKD patients[18]. However, already in this trial, it was established that many tolvaptan-treated patients deal with thirst (55.3%) and polyuria (38.3%), as well as several serious adverse events, such as elevations in alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin levels, indicative of hepatotoxicity[18]. This prompted a follow-up clinical study to determine the long-term safety and efficacy of tolvaptan in ADPKD patients, the TEMPO 4:4 trial, which displayed similar results as the first trial[178]. Also a third phase 3 clinical trial (REPRISE) was performed in late-stage ADPKD patients, confirming the results of both TEMPO trials, but also showed that tolvaptan treatment has no significant benefits in patients older than 55 years[20]. Thanks to these positive results, tolvaptan has been approved as an ADPKD drug in Japan, Canada, the European Union, Switzerland, South Korea and the United States. Due to the serious side effects regarding hepatotoxicity, the drug is only available to a subset of patients with rapidly progressing ADPKD[21]. This has also resulted in research on other vaptans, out of which lixivaptan was particularly interesting. Lixivaptan has been tested preclinically and can slow down disease progression in mice[85,175,179-181]. In addition, mathematical modelling predicted lixivaptan to have less risk for hepatotoxicity compared to tolvaptan. Two clinical trials have been started to assess the safety and efficacy of lixivaptan in ADPKD patients, but were recently terminated due to clinically significant liver enzyme elevations[182].

Somatostatin analogues

By binding to somatostatin receptors, somatostatin can inhibit AC-mediated cAMP synthesis in the collecting duct[182,183]. Therefore, long-acting somatostatin analogues such as octreotide and pasireotide have been tested preclinically in rodent models and were found to be effective in reducing cyst progression in both kidney and liver, with pasireotide outperforming octreotide[184-186]. These positive results were followed up by a large clinical trial (DIPAK-1) investigating the effect of long-term lanreotide treatment in ADPKD patients[187]. While TKV growth was reduced by lanreotide, no delayed decline in kidney function was observed. Clinical trials (ALADIN and ALADIN-2) investigating octreotide long-acting release (LAR) had similar findings[188,189]. A combined treatment of tolvaptan and octreotide has also been tested in a phase 2 clinical trials, showing that a combination treatment can enhance tolvaptan's beneficial effects on eGFR and TKV, in line with a previous preclinical outcome[186,190].

mTOR inhibitors

The mTOR pathway is a central pathway in the cell that integrates growth factor cues and nutrient availability in order to regulate cell proliferation and energy metabolism, through the mTORC1 and mTORC2 proteins[191]. It was found that mTOR pathway activity is regulated by PC-1, and that the pathway is overactivated in ADPKD[192]. This lead to a plethora of studies investigating mTOR pathway inhibitors, such as rapamycin/sirolimus and everolimus, in ADPKD, indeed showing that mTOR inhibition slows down disease progression in rodent models[192-198]. However, this effect could not be replicated in phase 3 clinical trials, possibly due to usage of a too low dose[199-202]. A possible option to increase the effective dose would be to administer a folate-drug conjugate, as folate receptors are highly expressed on the kidney epithelium[203]. Indeed, folate conjugates of rapamycin and dactolisib (a dual PI3K/mTOR inhibitor) accumulated in the kidney and slowed down disease progression in mice[203-205]. Importantly, no other organs were affected by folate conjugate drug treatment, which is the major concern for increasing the therapeutic dose of mTOR inhibitors for human use[206-208].

microRNA therapeutics

In recent years, a number of studies have shown that cystic disease progression can be reduced *in vivo* through microRNA (miRNA) therapeutics. miRNAs belong to the non-coding RNAs used in the post-transcriptional regulation of gene expression. Several miRNAs have been shown to regulate disease progression and subsequent intracellular pathway activity in ADPKD[209]. miR-21, miR193-3p and miR-214 have all been reported to have negative (miR-21) or positive effects (miR193-3p, miR-214) on cystic disease progression, but the best described in this regard is the miR-17~92 cluster[210,211]. The miR-17~92 cluster was first shown to be upregulated in *Kif3a*-KO mice and later as well in *Pkd1*-KO and *Pkd2*-KO mouse models, with the upregulation mediated by the oncogene cMyc[117,212]. Subsequent research showed that the miR-17 family is the primary driver of disease progression in ADPKD within the cluster, that miR-17 can bind to *Pkd1* and *Pkd2* through their 3'-untranslated region (UTR) miR-17 binding element, and that deleting or pharmaceutically blocking this element ameliorates disease progression in mice[120,213,214]. Blockade of the 3'-UTR miR-17 binding element was done with the anti-miR-17 oligonucleotide RGLS4326, which has shown to be non-toxic in both mice and primates and is currently in a Phase 1b clinical trial (NCT04536688)[215].

Dietary metabolic interventions

As discussed before, numerous metabolic processes are dysregulated in ADPKD, due to the Warburg-like metabolic reprogramming that takes place. As a consequence, more research in the last years has focused on various metabolic interventions, both dietary and pharmacological, in order to prevent disease progression. One type of dietary intervention, caloric restriction (reducing caloric intake), has wide-ranging benefits in terms of metabolic health, longevity and healthy aging [216,217]. To the contrary, being overweight or obese are detrimental to these processes, and have been found to be independent predictors of ADPKD disease progression and TKV growth[218,219]. In preclinical studies, moderate caloric restriction (10-40% reduction) in *Pkd1*$^{RC/RC}$ and *PKD*$^{cond/cond}$:Nes^{Cre} mice was already enough to reduce disease progression and altered cellular signalling[220-222]. A preliminary clinical trial among 15 ADPKD patients showed that an approximate 30% caloric restriction for 12 months is feasible in patients and results in weight loss correlating with slower kidney growth[222]. A new clinical trial with 2 year follow-up, comparing caloric restriction to standard advice control, is currently underway (NCT04907799). Several studies have looked into the effects of low protein and low fat diets, instead of full caloric restriction. In *pcy* mice, it was found that a low protein diet reduces disease progression and increases survival, however multiple clinical studies have since found no beneficial effects of a low protein diet[223-225]. In a similar vein, low fat diet-fed *Pkd1*$^{cko/cko}$ mice have reduced cystic parameters, while high fat diet-fed Han:SPRD-*cy* rats have increased cystic parameters)[116,226-228], but no dedicated clinical trial has been performed at this point. Caloric restriction diets are difficult to adhere to for patients, mainly due to lifestyle reasons[229]. A better option could be time-restricted feeding (or intermittent fasting), in which the access to food is time-restricted, but not calorie-restricted, which has been shown to prevent disease in rodents and humans[230]. Time-restricted feeding was found to improve disease parameters in Han:SPRD rats, but not in the *Pkd1*$^{RC/RC}$ mouse model[222,231]. A preliminary clinical trial among 13 ADPKD patients showed that intermittent fasting for 12 months also is feasible in patients and results in weight loss correlating with slower kidney growth[222]. A larger clinical trial investigating time-restricted feeding in ADPKD patients (29 patients, 24 month follow-up) is currently underway (NCT04534985). The positive time-restricted feeding results together with other parameters measured suggest a state of ketosis was induced in the rats[231]. Indeed, a ketogenic diet (high in fats, low in carbohydrates) ameliorated disease progression in young and adult Han:SPRD rats, as well as oral supplementation of the ketone β-hydroxybutyrate[231]. A recently published clinical trial testing the effects of a 5 month ketogenic diet in ADPKD patients shows that the diet induces weight loss and improves eGFR, but does not change TKV[232].

Pharmacological metabolic interventions

Many different options exist for pharmacological intervention on the Warburg-like metabolic reprogramming present in ADPKD. However, the most effective results would likely be yielded by targeting a protein/pathway that affects most of the dysregulated processes, i.e. excessive glycolysis, impaired FAO and altered mitochondrial function. One target that ticks these boxes is AMPK, one of the central regulators in cellular metabolism. AMPK, when active, regulates glucose transport by promoting translocation of glucose transporters 1 and 4 (GLUT1/4) to the cell membrane[233,234]. Lipid metabolism is regulated through the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (CoA) reductase (HMGR) and acetyl-CoA carboxylase (ACC), limiting fatty acid/lipid synthesis and promoting FAO[235-237]. AMPK can promote mitochondrial biogenesis through the activation of PGC1α, which as a transcriptional co-activator, interacts with among others, PPARα, to promote the transcription of genes involved in FAO, mitochondrial DNA transcription and replication[238,239]. In addition to this, AMPK activation results in the inhibition of cell growth and cell proliferation, through inhibition of mTORC1, phosphorylation of p53, as well as inhibition of the Hippo-YAP and RAS/B-RAF/MEK/ERK pathways[240-245]. Because of this versatility, AMPK activators are increasingly becoming attractive as potential ADPKD treatment, and have already been tested preclinically. Both caloric restriction and intermittent feeding modulate the intracellular AMP-ADP/ATP ratio (an important regulator of AMPK activity) and increase AMPK activity[220,221,231]. Compound-wise, the antihyperglycemic agent metformin has been the most researched. Originally used to treat type 2 diabetes, it indirectly activates AMPK by inhibiting mitochondrial complex I, which in turn affects the AMPK-modulating intracellular AMP-ADP/ATP ratio[246,247]. Preclinical data on metformin treatment in ADPKD is conflicting, with studies reporting that metformin slows down disease progression in mice, zebrafish and a miniature pig model, but there are also several studies (see chapter 3) that show no effect of metformin on ADPKD in mouse models[248-253]. Multiple clinical trials have been or are underway testing metformin as a therapeutic in ADPKD. The TAME-PKD phase 2 clinical trial investigated the safety, tolerability and efficacy of metformin in 21 ADPKD patients and found metformin to be safe and tolerable, while eGFR decline was non-significantly reduced[254]. A second trial in 22 participants also found no significant changes in TKV or eGFR, although this was not the primary outcome of the study[255]. Two larger phase 3 clinical trials with metformin are currently underway (NCT04939935, NCT03764605).

Several pharmacological metabolic interventions that are being investigated in ADPKD do not have AMPK as a direct target, but do affect its activation indirectly. One such compound is 2-deoxyglucose, which as a glucose analogue, can inhibit glycolysis, activate AMPK and reduce disease progression in different ADPKD models[102-104,250]. A pilot clinical study investigating the pharmacokinetics, safety and tolerability of 2-deoxyglucose is currently being planned[256]. A different group of compounds, the thiazolidinediones, are synthetic activators of the transcription factor PPARγ, which regulates fatty acid and glucose metabolism, cell proliferation, inflammation and fluid transport, in part through AMPK activation[257-261]. Several thiazolidinediones have been shown to reduce disease progression in *Pkd1*$^{-/-}$ embryos and orthologous rat models[262-265]. Based on these results, a small phase 1b clinical trial investigating the safety and efficacy of pioglitazone in 15 patients found pioglitazone to be safe for ADPKD patients. However, no significant change in TKV was observed[266]. The gliflozin class of medication are sodium/glucose cotransporter 2 (SGLT2) inhibitors, which can activate AMPK through their inhibitory function[267]. However, the drug canagliflozin had no effect on disease progression in an ADPKD mouse model (see chapter 3)[251]. Two clinical trials are currently recruiting participants to investigate the safety of empagliflozin administration in ADPKD patients (NCT06391450, NCT05510115). Other compound classes that have been tested were statins (pravastatin) and sirtuin inhibitors (niacinamide), which both were effective in preclinical or preliminary clinical studies, but showed no clinical benefit in larger clinical trials[268-271].

Other interventions

The retinoic acid receptor (RAR) is currently under investigation as a possible target in ADPKD, as it has been shown that the RAR agonist tamibarotene has anti-inflammatory and anti-fibrotic effects in unilateral ureteral obstruction (UUO) models of chronic kidney disease[272,273]. A phase 2 clinical trial investigating the effect of 1-year tamibarotene treatment in ADPKD patients is now recruiting participants (NCT06289998). In part due to the metabolic reprogramming in ADPKD, patients also suffer from increased body weight and insulin resistance[218,219,274]. Because of this, the recently approved class of glucagon-like peptide 1 (GLP1) antagonists, such as semaglutide and liraglutide, might be beneficial in ADPKD treatment. The effect of 22-month semaglutide treatment on cystic disease progression will be investigated in a phase 2 clinical trial (NCT06582875).

Aim and outline of this thesis

ADPKD is a progressive, genetic kidney disease, affecting about 10 million individuals worldwide, and accounting for 5-10% of ESRD cases worldwide. Despite a large amount of preclinical and clinical investigations, thus far, only one drug has been approved for the treatment of ADPKD: tolvaptan. However, due to its numerous side-effects (such as polyuria and hepatotoxicity), only a subset of patients can benefit from its use, and therefore, there is still a large unmet clinical need for therapeutics. In this thesis, we aim to identify novel therapeutics, and test them in preclinical models, to evaluate their safety and most importantly, their efficacy in slowing down disease progression, with the end goal of providing new candidates for clinical investigation.

In **chapter 2**, we investigate whether a combination treatment of tolvaptan and the thiazolidinedione pioglitazone can improve clinical efficacy compared to single-drug treatment in an adult-onset PKD mouse model. The combination treatment improved renal survival and slowed disease progression, but it wasn't more effective than tolvaptan alone. Unexpectedly, pioglitazone treatment had no effects on these parameters, although systemic biomarkers were affected by pioglitazone.

In **chapter 3, 4 and 5**, we perform a thorough investigation of the direct AMPK activator salicylate and its prodrug salsalate. We find that salsalate can slow disease progression and improve renal survival in an adult-onset PKD mouse model, in contrast to indirect AMPK activators metformin and canagliflozin (chapter 3). We then delineate with the use of *in vitro* models through which targets and pathways salicylate could exert its beneficial effects, and whether those effects could be AMPK-independent (chapter 4). Next, we validate the positive effects of salsalate *in vivo* with a new experimental setup, designed to eliminate potential phenotypic biases (chapter 5).

In **chapter 6**, we follow-up on the main results from a previously published 3D drug screening of ADPKD therapeutic candidates. Our main hit, fiboflapon, was found to reduce cyst swelling in both murine and human ADPKD 3D cysts. We followed this up with an *in vivo* study of fiboflapon in a progressive, early-onset ADPKD mouse model characterized by rapid disease progression. Fiboflapon did not affect disease progression or relevant cystic parameters, but it did affect its predicted drug marker in blood.

Finally, in **chapter 7**, we present a general overview and discussion of our obtained results and provide suggestions for future research.

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