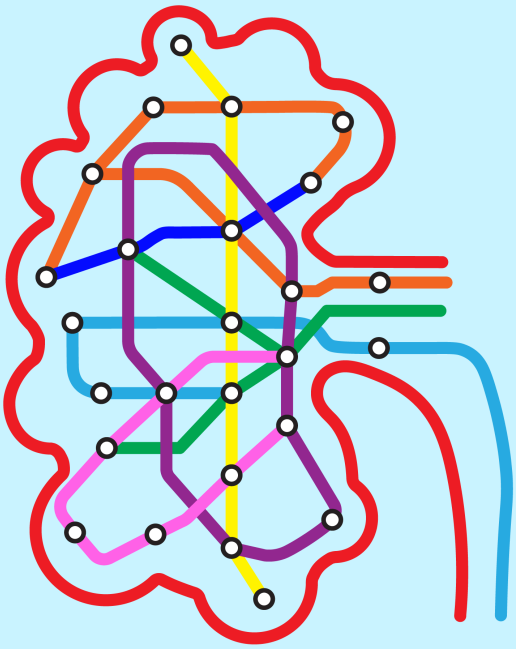
Preclinical validation and mechanistic understanding of drug repurposing candidates for polycystic kidney disease

Kanhai, A.A.

CHAPTER 7

Summarizing discussion



The exact mechanisms linking cyst formation and disease progression in autosomal dominant polycystic kidney disease (ADPKD) remain to be fully understood, although significant progress has been made in recent years. Much more is known about the consequences of cyst growth: dysregulation of the intracellular signaling machinery. Many therapeutic candidates have been tested preclinically and also in clinical trials, but so far only the vasopressin V2 receptor antagonist tolvaptan has been approved as an ADPKD treatment. Side effects of tolvaptan use include hepatotoxicity and polyuria, which limits its use to only a subset of patients with rapidly progressing ADPKD[1]. A large group therefore remains without effective treatment.

Due to the high costs involved in the development of novel drugs, drug repurposing, the use of known drugs for new indications, has become more attractive. Drug repurposing has already yielded positive results, with a recent prominent example being the antiviral remdesivir named first-in-class treatment for COVID-19[2,3]. As many pathways are affected by disease progression in ADPKD, the list of drug targets viable to target via drug repurposing is extensive. In this thesis, we have investigated various drug treatments originally developed for other indications, for their efficacy in preclinical ADPKD models. In addition, we have looked into the molecular mechanisms involved in their potential effects.

In **chapter 2**, we investigated the preclinical potential of a combination treatment of tolvaptan and the PPARγ agonist pioglitazone. As both have been shown to slow down disease progression *in vivo* via different pathways, we hypothesized that a combination treatment could be synergistic and more effective than single-treatment with either drug[4-6]. Both tolvaptan single-treatment and the combination treatment were effective, but comparable in improving renal survival and in slowing down disease progression in our adult-onset *Pkd1* deletion mouse model (iKspCre-*Pkd1*^{del}). However, we did not see any positive effects of pioglitazone single-treatment on renal survival, 2KW/BW% and the cystic index. While the chosen dose, 30 mg/kg/day, was ineffective, plasma adiponectin levels were similar to healthy individuals and type 2 diabetes patients treated with pioglitazone[7,8]. Our data is in line with a previous mouse study, which also did not observe any beneficial effect of pioglitazone treatment, albeit a younger mouse model and a lower dose was used[9]. A possible reason behind the differences in pioglitazone efficacy across models is the expression of its target PPARγ in the kidney. We observed a very low PPARγ protein expression in the mouse kidney and confirmed that this expression level was conserved in rat and human kidney samples. In spite of this low expression, previous preclinical studies in orthologous PKD rat models found positive effects of pioglitazone and its sister compound rosiglitazone on cystic disease progression using a subclinical dose[4,6,10-12]. Although the results of pioglitazone in orthologous PKD rat models show a positive effect on cystic disease progression, both studies are underpowered and report limited results, making it difficult

to draw proper conclusions. The positive results in the rat models prompted a clinical trial (NCT02697617) in which ADPKD patients were treated with low-dose pioglitazone[13]. The safety endpoints showed no differences between treatment and placebo, although longer studies are required to properly evaluate drug safety. Efficacy endpoints showed no effects of pioglitazone treatment on total kidney volume (TKV), kidney cyst volume, kidney cyst index and the estimated glomerular flow rate (eGFR) after two years of treatment. As the study was hindered by a low sample size, also drug efficacy should be evaluated in a longer follow-up study.

Nevertheless, the preclinical studies on pioglitazone show a discordance in results, possibly due to differences in pioglitazone target expression, pharmacokinetics, pharmacodynamics and extra-renal manifestations between the species. Although further clinical investigations are required to establish pioglitazone's clinical efficacy, the seemingly contrary research on pioglitazone in ADPKD illustrates the number of and the heterogeneity within *in vivo* models used in preclinical research, and the difficulties comparing results between them. The translation of preclinical results is highly dependent on for example, the species, age, genetic background, disease onset and disease progression of the chosen model. The high number of tested compounds that do not show clinical efficacy reinforces the notion that every preclinical model has its limitations in mimicking human disease and that a more standardized approach in the preclinical validation of ADPKD therapeutic candidates is required. A first step towards this could be to implement a recently proposed standardized preclinical workflow[14]. This can then be expanded to the testing of treatment candidates in at least two animal models, out of which at least one has a *Pkd1* mutation related to human disease to ensure translatability[15,16]. Clinical translation can be aided further by including novel tools and human *in vitro* models in testing, such as ADPKD patient-derived induced pluripotent stem cells, CRISPR/Cas9 technology, ADPKD patient-derived 3D cultures, kidney organoids and tubuloids and organ-on-a-chip technology[17-22].

In **chapter 3**, we investigated whether AMPK activation can be effective in reducing cystic disease progression *in vivo*. To achieve this, we used the anti-diabetic drug metformin, previously shown to slow down cystogenesis in mice and a miniature pig model[23,24]. We also tested two other AMPK activators, canagliflozin and salsalate, as well as a combination treatment of metformin with each of these compounds, to examine potential additive or synergistic effects. We found that mice treated with salsalate (single treatment or in combination with metformin) have attenuated renal cystic disease, while groups treated with canagliflozin and/or metformin showed no improvement. We followed up on this research to further detail the molecular mechanisms involved in salsalate's beneficial effects.

In **chapter 4**, we investigated the effects of a short salsalate treatment (2 weeks) on mild cystic mice, to have limited interference of the dysregulated intracellular signalling at later stages of the disease. Using NMR metabolomics and bulk RNA-sequencing, we found that salsalate reverts the metabolic reprogramming in ADPKD and also reduces the increased proliferative and inflammatory signalling in ADPKD. Salsalate is a prodrug of salicylate, a compound that activates AMPK, but also has been reported to have other binding partners.

We therefore investigated in **chapter 5** whether the effects of salicylate on ADPKD signalling pathways *in vitro* are attributable to AMPK activation alone, or whether other salicylate binding partners also have a role in this. We found that salicylate affects cellular metabolism, cell proliferation, inflammation and oxidative stress in *Pkd1*$^{-/-}$ renal epithelial cell lines via AMPK activation, but also can induce mitochondrial uncoupling independent of AMPK. In addition, we found salicylate reduces forskolin-induced cyst swelling in both murine and ADPKD patient-derived 3D cultures.

AMPK is classically viewed as a master regulator of cellular energy homeostasis, ensuring an ATP balance by increasing generation and decreasing usage. However, it has many more functions, also regulating autophagy, mitochondrial and lysosomal homeostasis, cell proliferation, DNA repair, cytoskeletal remodelling and cellular immunity[25]. Therefore, it is more apt to view AMPK as not only a regulator of energy homeostasis, but as a regulator of cellular homeostasis in general. Its central role also illustrates its fitness as a therapeutic target in chronic diseases like ADPKD, as many of these processes mentioned are dysregulated in these conditions. Earlier research on AMPK targeting in ADPKD revealed metformin can slow down cyst growth in 3D cultures and two *Pkd1* deletion mouse models[23]. This was followed by studies in other animal models and a phase 2 clinical trial in ADPKD patients[24,26-28]. While the trial found only non-significant effects on eGFR (improved) and height-adjusted TKV (worsened), 65% of metformin-treated patients had to discontinue the medication or adjust their dose regimen, indicating that the chosen dosage (2000 mg/day), although safe, might not be feasible for long-term treatment. However, the pharmacokinetics of metformin dictate that the used dosage is required for therapeutic renal efficacy[29,30]. We therefore reasoned that by using a second AMPK-activating drug in combination with metformin, we could improve therapeutic efficacy. We found salsalate treatment to be effective in slowing down cystic disease progression, but both canagliflozin and metformin were not effective. Metformin inefficacy is contrary to previous results and could be due to differences in administration. We opted for oral administration versus intraperitoneal administration in the earlier mouse study. Another reason for the discordance could be the rate of disease progression in the models. As we inactivate *Pkd1* at post-natal (PN) day 18 and 19, this results in a slow disease progression (renal failure at 4 months), similar to human disease progression[31]. Our results are in line with that of the slowly progressive

Pkd1$^{RC/RC}$ model, in which also no beneficial metformin effects were seen[32,33]. In contrast, the earlier mouse study inactivated *Pkd1* at PN 9-10, resulting in a faster disease progression (renal failure at 2 weeks). Moreover, the miniature pig model in which metformin showed efficacy is also a slow progressive disease model, but used a human equivalent dose of 2.77 g/day, which is not feasible for human use[24,34]. These differences in study setup again illustrate the need for a more standardized approach to preclinical testing of therapeutic candidates. As the phase 2 clinical trial for metformin showed potential, a phase 3 clinical trial (IMPEDE-PKD, NCT04939935) is currently recruiting participants, and will be using a slow-release formulation of metformin, possibly circumventing the problems with regular oral metformin administration. Moreover, another clinical trial is currently underway testing a sister compound of canagliflozin, empagliflozin (EMPA-PKD**,** NCT06391450)[35]. The results of both clinical trials will provide more insight whether AMPK-activating therapeutics are suited for ADPKD treatment.

We further investigated the molecular mechanisms through which salsalate exerts its beneficial effects in cystic disease. As our main goal in chapter 3 was to test efficacy and not look into the molecular mechanisms involved, it is prone to phenotypic bias. Because cystic mice are treated with a compound that slows down cystic disease progression, these mice will have a less severe phenotype and cellular signalling pathways that are less dysregulated than their untreated cystic counterparts. However, as the disease phenotype is less severe, it is impossible to distinguish compound-specific signalling effects, as any detected effects on molecular signalling are likely due to the less severe phenotype, rather than effects of the treatment. We therefore opted for a novel *in vivo* experimental design in **chapter 4**,in which we chose to treat only mildly cystic animals with salsalate for a relatively short time period, and compare them to untreated cystic animals with a similar disease phenotype. In this way, any detected changes can be directly attributed to salsalate. We found that salsalate activates fatty acid oxidation in mildly cystic mice, likely due to its AMPK-activating capabilities. In addition, both NMR metabolomics and bulk RNA-sequencing data indicate that salsalate also inhibits inflammatory, proliferative and oxidative stress pathways and processes *in vivo*.

While many of these beneficial effects are possibly regulated through salsalate-induced AMPK activation, another option is that these effects are due to possible secondary targets of salicylate, salsalate's active compound. In line with this possibility, multiple salicylate binding partners have been established in literature, most of which are often involved in cystic disease mechanisms[36]. We therefore looked in **chapter 5** whether salicylate effects on ADPKD signalling *in vitro* were solely mediated through AMPK or also through secondary salicylate binding targets. We found that for most of the targets, salicylate exerts its effects mainly via AMPK activation. Main features of ADPKD pathogenesis, such as dysregulated

energy metabolism, epithelial cell hyperproliferation, higher pro-inflammatory activity and increased oxidative stress were all affected by salicylate treatment in proximal tubule and collecting duct *Pkd1*$^{-/-}$ cell lines, as well as by the direct AMPK activator A-769662 and the indirect AMPK activator AICAR. This confirms the mechanisms found *in vivo* in chapter 4 and as all AMPK activating compounds affect the pathways and processes in a similar manner, the AMPK-activating effect of salicylate/salsalate appears to be the driving force behind its beneficial role in ADPKD *in vitro* and *in vivo*. We however also found an AMPK-independent salicylate characteristic, namely mitochondrial uncoupling. Together with the AMPK-dependent slowdown of glycolysis, it appears that salicylate/salsalate shift the metabolic state of cystic epithelial cells from an anabolic one to a catabolic one through the re-activation of less active metabolic pathways such as fatty acid oxidation. This metabolic shift by lower glycolytic activity and higher oxidative activity has been reported before, both in preclinical and clinical studies[37-44]. To translate our findings to a human model, we also tested whether salicylate reduces 3D cyst growth in ADPKD patient-derived tissue. We indeed found that salicylate reduces cyst growth in this model in a non-toxic manner, showing for the first time that salicylate/salsalate is effective in reducing cyst growth in an ADPKD model derived from patient tissue.

The beneficial effects of salicylate/salsalate likely extend further than only targeting the cystic epithelium. As an aspirin derivative, salsalate holds similar anti-inflammatory properties, but essentially lacks the acetyl moiety responsible for the bleeding risks and gastrointestinal side-effects associated with aspirin[45]. An increased inflammatory response is an ADPKD hallmark, providing another disease mechanism for salsalate to target. Indeed, we show in chapters 3, 4 and 5 that salsalate treatment reduces the pro-inflammatory effects *in vitro* (reduced activity of the pro-inflammatory NFkB pathway) and *in vivo* (less macrophage infiltration and reduced pro-inflammatory gene expression) through AMPK activation. AMPK activation has been shown before to reduce the inflammatory response observed in chronic disease models of atherosclerosis, colitis, hepatic injury and arthritis[46]. In addition, salsalate administration in diabetic patients and obese individuals has been shown to reduce circulating levels of C-reactive protein (CRP), soluble CD40 ligand (sCD40L) and immune cell count[41,43,47]. When also taking into account that salsalate has already been approved by regulatory agencies, partly eliminating the high clinical testing costs, the case is strengthened for salsalate to be further developed as a ADPKD drug. A first step towards this should be to test whether salsalate can also reduce cyst growth in a second *Pkd1* mutation mouse model, such as the *Pkd1*$^{RC/RC}$ mouse model[48,49].

We followed up on an earlier drug repurposing study in **chapter 6**, where we tested the 5-lipoxygenase activating protein (ALOX5AP or FLAP) inhibitor fiboflapon, one of the most promising hits of a previously performed drug repurposing screening combining gene

expression profiling, bioinformatics, and cheminformatics[50]. We found fiboflapon reduces cyst swelling in both mouse collecting duct and ADPKD patient-derived 3D cysts, but did not slow down cystic disease progression in a progressive and early-onset tamoxifen-inducible *Pkd1* deletion mouse model. Moreover, fiboflapon did not alter changes in renal health, cystic severity, renal fibrosis and macrophage infiltration induced by the cystic phenotype. FLAP is part of the leukotriene pathway, which generates pro-inflammatory signaling molecules (leukotrienes) mainly in inflammatory cells such as neutrophils and macrophages[51]. As such, leukotriene pathway activity is relatively low in the epithelium, and it is therefore interesting that we observe cyst reducing effects of fiboflapon in 3D cyst assays, where no immune cells are present. Our results imply that either secondary fiboflapon targets are responsible for the *in vitro* cyst reducing effects or that FLAP has non-leukotriene related interactions. Secondary fiboflapon targets have not yet been identified, in part due to the lack of publicly available preclinical data on fiboflapon. On the contrary, proteomic studies in human tissue indeed identify potential FLAP interactions relevant to ADPKD[52,53]. One such interactor is the chloride intracellular channel 1 (CLIC1), which is highly expressed in renal proximal tubule cells[54]. CLIC1 might be involved in ADPKD pathogenesis, as chloride secretion has been described as a driver of cyst growth[55]. In addition, CLIC1 knock-out reducing oxidative stress and tissue fibrosis in mice, which are two other ADPKD pathogenic mechanisms[56,57]. Another suggested interactor is aquaporin 6 (AQP6), belonging to the aquaporin family of water channels. It therefore might play a role in fluid secretion into the cyst lumen, although further studies are needed to validate this. Lastly, the free fatty acid receptor 2 (FFRA2), also known as G-protein coupled receptor 43 (GPR43) is a suggested target, potentially linking FLAP to the dysregulated metabolism in ADPKD. FFRA2/GPR43 is expressed in the collecting duct and is mainly activated by short-chain fatty acids (SCFAs) such as acetate and butyrate[58,59]. FFRA2/GPR43 activation promotes the use of fatty acids in non-adipose tissues, re-activating fatty acid oxidation, possibly beneficial in the context of ADPKD[60]. In addition, multiple studies show FFRA2/GPR43 activation protects against diabetic nephropathy and attenuates chronic kidney disease *in vivo* by reducing pro-inflammatory, pro-fibrotic and pro-oxidative stress pathways[61-64]. While the exact interactions between FLAP and CLIC1, AQP6 and FFRA2 remain to be elucidated, the possible benefits of targeting FLAP in ADPKD warrant future studies. These future studies should first focus on testing fiboflapon efficacy in an adult-onset, slow progressive ADPKD model with a dosing regimen comparable to patient treatment.

Future perspectives

Current research on ADPKD mainly investigates whether it is possible to slow down disease progression, rather than investigating possible curative options. This is a prudent approach, as the diversity in polycystin mutations in ADPKD is very large, making therapy development for a large group of patients very difficult. In addition, due to the polycystin protein size,

the structural complexity of cell membranes and the delivery of proteins to the right tissue, it is virtually impossible to test polycystin protein replacement therapies in current model systems. However, steps forward have been made in restoring deleted polycystin genes, as a recent study showed that PKD in mice can be reversed fully by *Pkd1* or *Pkd2* re-expression in an early disease stage and partially in a late disease stage[65]. Another study using organoids from human pluripotent stem cells with nonsense *PKD1* or *PKD2* mutations showed that heterozygous mutants do not develop cysts, while homozygous mutants do[66]. Base-corrected (either by adenine or cytosine base editing) heterozygous mutants also showed no cyst growth *in vitro*. In addition, treatment of homozygous mutants with eukaryotic ribosomal selective glycosides (ERSGs, compounds enabling ribosomal readthrough of nonsense mutations) resulted in polycystin re-expression as well as reduced cyst growth. Remarkably, ERSG treatment even resulted in cyst reversal in individual cultures. Both studies are examples of the regenerative capacity of the cystic kidney, but it remains unclear which mechanisms and/or cell types are responsible for the cyst reversal. Utilizing these possible disease-curing applications clinically will likely take decades, but this research is nevertheless a first step towards understanding cyst reversal mechanisms.

Many options are available and have been tested for treating ADPKD, but so far only the vasopressin V2 antagonist tolvaptan has been approved for clinical use in ADPKD patients. As already discussed, a more ordered approach towards preclinical testing, using a standardized workflow and adult-onset, slow progressive *Pkd1* mutation models will likely benefit translatability towards the human situation. As many signalling pathways are affected, also a plethora of targets is available, making a multitarget approach viable through either a combination treatment of multiple drugs (as we pursued in chapters 2 and 3) or by using a drug with multiple putative targets, such as salicylate/salsalate (as we pursued in chapters 4 and 5). Both options target the signalling machinery from different angles. Moreover, a combination treatment could result in possible synergism between the two drugs, increasing clinical efficacy and possibly enabling clinicians to use lower drug doses, limiting unwanted side effects. In the clinic, a possible combination therapy was tested by comparing tolvaptan monotherapy to tolvaptan and octreotide long-acting release (LAR). The combination of tolvaptan plus octreotide-LAR lowered both total and cystic kidney volumes, compared to tolvaptan monotherapy[67]. Although only performed in a small group of patients, this study demonstrates combination treatments are feasible in ADPKD and warrant more research into the different signalling pathways that could be targeted simultaneously for a larger therapeutic effect.

The majority of therapeutic ADPKD research has focused on the cystic epithelium as a target, as those cells are primarily affected by cyst formation and do not function properly as a result of cyst formation. However, other cells play an important role in cyst formation

and cyst growth as well, in particular immune cells. Macrophages have been described quite extensively in ADPKD, but until recently, other innate and adaptive immune cells have rarely been investigated. In the last years, the role of the adaptive immune system in ADPKD is becoming more clear, with multiple reports demonstrating the pro-cystic role of $CD4^+$ T-cells and the cyst-protective role of $CD8^+$ T-cells[68,69]. The role of other adaptive immune cells and (sub)populations needs to be further studied, which will be aided by a recent investigation performing single-cell RNA-sequencing of $CD45^+$ immune cells in the mouse cystic kidney. This study showed that adaptive immune cell subtypes present in non-cystic controls, non-injured aged cystic mice and injury-accelerated cystic mice are significantly different[70]. Moreover, *Rag1*-deficient cystic mice (lacking an adaptive immune system) showed attenuated cystic disease in injury-accelerated cystic mice, but not in aged cystic mice. This is in line with previous results that showed renal injury accelerates cystic disease progression in both mice and humans, and has been suggested to be a 'third hit' required for cyst development in ADPKD[71]. Immune cells contribute to the propagation of the injury by the production of pro-inflammatory cytokines through inflammatory pathways, such as the NFkB and JAK-STAT pathways, which are consequently upregulated in ADPKD[72]. Considering the effectiveness of immunosuppressive therapies in other chronic diseases, further research into applying these could benefit ADPKD patients. Indeed, combination treatment with multiple immune checkpoint inhibitors in adult-onset *Pkd1*$^{RC/RC}$ mice resulted in improved disease outcomes[73]. Although caution is required due to the long-term treatment that is required for ADPKD, this study is an example of how targeting of non-epithelial signalling might be beneficial in search of an ADPKD treatment.

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