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**Author:** Hassane, Sabine

**Title:** Renal cyst and aneurysm formation in polycystic kidney disease mouse models

**Issue Date:** 2012-03-20

**Renal cyst and aneurysm formation  
in Polycystic Kidney Disease  
mouse models**



**Renal cyst and aneurysm formation  
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Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,

volgens besluit van het College voor Promoties

te verdedigen op dinsdag 20 maart 2012

klokke 15.00 uur

door

Sabrine Hassane

geboren te Koeweit

in 1978

## **Promotiecommissie:**

Promotor: Prof. Dr. M.H. Breuning

Co-promotor: Dr. D.J.M. Peters

Overige leden: Prof. Dr. M.C. de Ruiter  
Prof. Dr. J. Drenth  
Dr. E. de Heer

Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

*And whoever saves it (a soul) is as if he had saved mankind entirely.*  
*Holy Quran 5:32*



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# Chapter 1

## Introduction

## Autosomal Dominant Polycystic Disease

Autosomal Dominant Polycystic Disease (ADPKD) is the most common genetic disorder causing chronic kidney disease with a prevalence of 1:400-1:1000.<sup>1</sup> Affected patients have numerous fluid-filled cysts in the kidneys. Over time, the cysts progressively increase in size and become surrounded by fibrosis. The enlarging cysts compress surrounding normal nephrons resulting in a decline of renal function. It is a typically adult onset disease first manifesting after the age of 30, though occasionally it manifests as an early onset disease in infants.<sup>2-4</sup> Most patients develop end stage renal failure (ESRF) in the fifth or sixth decade. Besides ESRF, ADPKD patients may suffer from other complications, like cysts in the liver, the pancreas, abnormalities in the arterial blood vessels as well as hypertension and renal adenoma.<sup>5</sup> About 60% of the patients have back, flank, or abdominal pain that may lead to physical impairment.<sup>6</sup> This pain may be due to kidney enlargement. Other causes of pain are cystic hemorrhage, infection, or renal stone. There is no cure for ADPKD and no therapy is yet clinically proven to retard cyst enlargement.<sup>7</sup>

## Genetics

In 85% of ADPKD patients, mutations are found in the *PKD1* gene and 10-15% of the cases in the *PKD2* gene.<sup>8</sup> The *PKD1* gene is located on chromosome 16 and spans 52 kb containing 46 exons.<sup>9</sup> The *PKD2* gene is found on chromosome 4 and is a smaller gene spanning 68 kb of genomic DNA and containing 15 exons.<sup>10,11</sup> A high level of allelic heterogeneity is found for both genes.<sup>12</sup> The fact that *PKD1* gene is more prone to mutations may be explained by the presence of several duplicates of the first part of the gene, which show 95-97% homology to the *PKD1* gene. These pseudogenes probably promote gene conversion.<sup>13</sup> Moreover, long polypurine-polypyrimidine tracts are present, which are capable to form multiple non-B-DNA structures and *PKD1* gene is larger than *PKD2* gene.<sup>14</sup> All these factors may predispose the gene to mutagenesis.

The ADPKD patients show widespread variations in disease severity ranging from rare cases showing massively enlarged cystic kidneys in utero,<sup>4,15</sup> through more typical presentation with end stage renal disease (ESRD) in the fifth or sixth decade, to cases with sufficient kidney function until old age.<sup>12,16</sup> Some studies indicate that the location of the mutation in the *PKD1* gene may influence the severity of the disease.<sup>17-19</sup> Patients with mutations in the 5' region of *PKD1* have more severe disease and are more likely to have intracranial aneurysms than patients with 3' mutations. Also patients with mutation in *PKD2* show a milder phenotype compared to the *PKD1* disease. This is due to the development of more cysts in *PKD1* disease at an early age.<sup>20,21</sup> Furthermore, environmental factors, smoking and diet have shown to influence the progression towards chronic renal failure.<sup>12,22</sup> However, variation especially found between family members, may also be due to modifying genes which might directly affect the function of polycystins, cyst formation or the clinical factors associated with disease progression.<sup>23</sup> Recent data suggest that also missense variants in the other allele can modify the phenotype.<sup>24</sup>

Several studies show that renal cysts may develop from loss of heterozygosity. However, dosage reduction (<20%) of the protein in two *Pkd1* animal models with hypomorphic alleles indicates that cysts can develop even if the protein is not completely lost.<sup>25,26</sup>

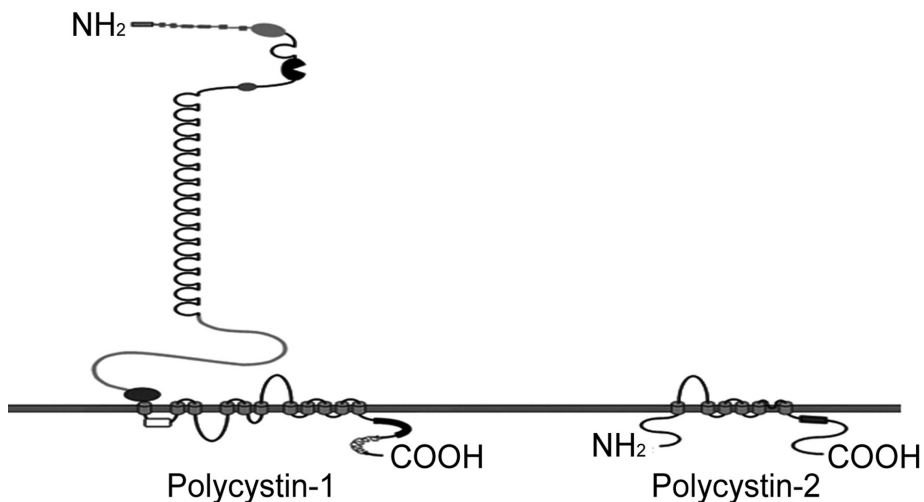
Furthermore, in transgenic mice overexpressing the *PKD1* and *PKD2* transgenes in the kidneys revealed renal cystic disease comparable to the human ADPKD phenotype.<sup>27,28</sup> These studies suggest that an imbalance in the expression of polycystins affects their function and lead to the development of PKD.

### Polycystins

The gene products of the *PKD1* and *PKD2* are polycystin-1 (PC1) and polycystin-2 (PC2), respectively (Figure 1). PC1 consist of 4302 amino acids weighting 450 kDa.<sup>29</sup> It has the structure of a receptor or adhesion molecule with a large extracellular N terminal segment of 3000 amino acids, 11 transmembrane domains and a relatively small intra-cellular C terminus, which has a coiled coil domain. The extracellular N terminus contains many domains, which are also present in receptors that mediate cell-cell and cell-matrix interactions suggesting the same function for PC1.<sup>30,31</sup> Furthermore, PC1 is involved in mechano-sensing and cellular signaling.<sup>32-34</sup> PC1 interacts with PC2 through a coiled-coil domain in the C-terminal portion and with multiple other proteins at different extracellular and intracellular sites.<sup>35</sup>

The extracellular part of PC1 contains a G-protein-coupled receptor proteolytic site. Cleavage at this site results in a C-terminal fragment and an N-terminal fragment. *Pkd1*-null mice die in utero, but mice with a mutation preventing cleavage of PC1 protein survive to post natal day 28 with enlarged cystic kidneys.<sup>36-40</sup> This may mean that cleaved PC1 is critical for maintenance of tubular integrity. In addition, proteolytic cleavage of the cytoplasmic carboxy-terminal part of the protein generates fragments directly involved in signal transduction.<sup>41</sup> PC1 is expressed during development in many organs e.g. kidneys, pancreas, liver, lung, intestines, brain, reproductive organs, placenta, thymus, hart, skeletal muscle, and blood vessels.<sup>42,43</sup>

**Figure 1. Predicted protein structure of polycystin-1 and polycystin-2.**



After birth the expression of PC1 decreases in the kidney except for the collecting ducts and distal part of the nephrons, which show the highest expression level of PC1 in the kidney.<sup>43,44</sup> The subcellular localization of PC1 is apical at the plasma membrane, at cell adhesion complexes, desmosomes, and the primary cilium.<sup>34,45</sup>

PC2 contains 968 amino acids with a molecular weight of 110 kDa. It has homology to the last six transmembrane segments of PC1.<sup>46</sup> The C-terminal part contains a calcium-binding motif (EF-hand) and coiled-coil domain, which may interact with the coiled coil domain of PC1 as well as other proteins.<sup>35,47</sup> It is also a hexaspanner protein, which resembles the  $\alpha$ -subunit of voltage-activated calcium and sodium channels. Therefore, it is suggested that PC2 is an ion channel especially permeable to calcium ions.<sup>46</sup> PC2 has been shown to localize predominantly to the endoplasmic reticulum, but also to the plasma membrane, primary cilium, centrosome, and mitotic spindles in dividing cells.<sup>34,48,49</sup>

PC1 and PC2 are expressed in almost the same tissues. However, the expression of PC2 does not decrease after birth and expression in adult kidneys is overlapping but not identical to PC1 expression, i.e. PC2 is mainly present in the medullary thick ascending limb and distal cortical tubules.<sup>42,50</sup>

### **Pathogenesis of Cyst Formation**

The polycystins are essential to maintain the normal structure of the tubular epithelium in the kidney. Reduction or increase of one of these proteins below or above a critical threshold results in cystogenesis, characterized by proliferation of the epithelium, excessive secretion of solute and fluid into the cysts, remodeling of the extracellular matrix and aberrant mechano-sensing via the cilia. The exact molecular mechanisms responsible for these phenotypic changes are not known, but our knowledge is increasing.

### **Proliferation and Apoptosis**

In normal human kidneys, renal tubular epithelial proliferation is strongly reduced soon after birth. In adult kidneys, cell proliferation is mainly seen during tissue repair after ischemic or toxic damage. In ADPKD kidneys, proliferation is increased in the cyst-lining epithelia.<sup>51</sup> Several studies suggest that proliferation accelerates the cyst formation during kidney development or tissue repair after injury. Conditional knockout mice of *Pkd1* at various time points have shown that the timing of *Pkd1* inactivation determines the progression of the disease.<sup>52,53</sup> Kidney development in newborn mice is not yet completed and epithelial cells show an increased proliferation rate compared to adult kidneys. Inactivation of *Pkd1* in the early neonatal period results in rapidly progressive cystic disease, whereas later inactivation causes much slower cyst development. *Pkd1* inactivation in adult mice, results in the slowest onset of cyst formation. This suggests that a certain threshold of cell proliferation must be surpassed. Furthermore, renal injury accelerates cyst formation in the kidneys of *Pkd1*-deletion mice and *Kif3a* knockout mice.<sup>54,55,56</sup> These findings suggest that cyst growth in adults with PKD may be affected by environmental factors such as kidney infection, or exposure to toxins. Individual variation in exposure to these environmental factors may contribute to the variation in severity of PKD that is observed between members of the same family who have inherited the identical gene mutation.<sup>22</sup>

Many pathways that are involved in epithelial cell proliferation are activated in PKD, including, mitogen-activated protein kinase/extracellular regulated kinase (MAPK/ERK) and mammalian target of rapamycin (mTOR).<sup>57,58</sup> In addition, several studies suggest that the polycystins can directly regulate the cell cycle. PC1 was reported to activate JAK2/STAT-1 signaling and upregulate p21 (a cell cycle inhibitor).<sup>59</sup> PC2 was reported to bind Id2, and prevent its translocation to the nucleus and suppression of p21, thereby activating cell cycle progression.<sup>60</sup> Indeed, p21 levels have been found to be decreased in human and animal PKD tissues.<sup>61</sup> Furthermore, *Pkd1* prevents immortalized proliferation of renal epithelia through the induction of p53 and the activation of JNK signaling.<sup>62</sup> Increased levels of cAMP are a common finding in the kidneys of many PKD models.<sup>35,63</sup> While under normal conditions cAMP inhibits mitogen-activated protein kinase signaling and cell proliferation, in PKD or in conditions of calcium deprivation it stimulates cell proliferation. The abnormal proliferative response to cAMP is directly linked to the low intracellular  $\text{Ca}^{2+}$  concentrations, which is suggested to be directly linked to deletion or overexpression of polycystins.<sup>57,63-66</sup> Upregulation of the vasopressin V2 receptor and high circulating vasopressin levels may contribute to the increased cAMP levels.<sup>67,68</sup> Furthermore, a bioactive lipid with the same biochemical and biological properties as forskolin, a potent adenylyl cyclase agonist, has been isolated and identified within the cyst fluid.<sup>69</sup>

Several studies have shown that increased tubular cell proliferation go along with increased apoptosis in PKD.<sup>58,70,71</sup> Apoptosis was detected in kidneys of humans with ADPKD regardless of renal function, but not in normal kidneys.<sup>51,71</sup> In these studies, apoptosis was detected in cystic and noncystic tubules in human polycystic kidneys suggesting that it may be associated with the progressive loss of normal nephrons in PKD. Furthermore, several *Pkd* models show increased apoptosis like, *Pck* and *Han:SPRD* rats.<sup>72,73</sup> Deletion of anti-apoptotic genes *Bcl-2* and *AP-2a* as well as overexpressing the pro-apoptotic *c-Myc* (SBM mice) results in renal cysts formation.<sup>74-76</sup> Other studies show a link between *PKD1* and apoptosis: the *Pkd1* hypomorphic mouse *Pkd1*<sup>L3/L3</sup> showed increased apoptosis and overexpression of *PKD1* has been shown to induce G0/G1 phase arrest in cell cycle and an increased apoptosis in cancer cell lines.<sup>26,77</sup> Furthermore, PC1 knockdown in MDCK cells is associated with increased rates of proliferation and apoptosis. This is accompanied by resistance to anoikis, inducing apoptosis by loss of cell anchorage.<sup>78</sup>

### Extra-Cellular Matrix Remodeling

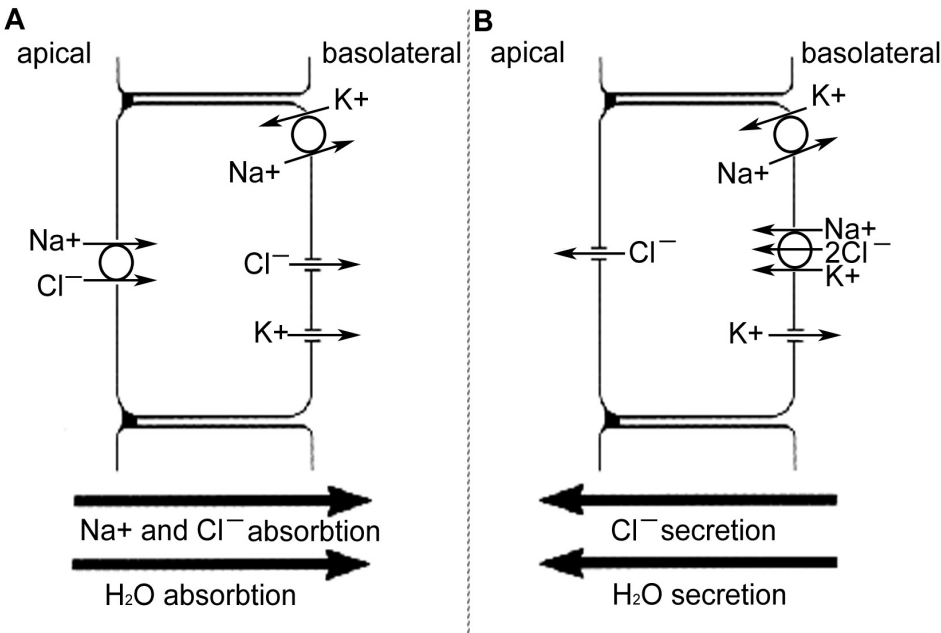
Renal interstitial fibrosis is common in human and animal models of PKD. It is characterized by infiltration of inflammatory cells, fibroblasts, and an abnormal accumulation of extracellular matrix (ECM) components resulting into progressive loss of normal renal tissue. Many of these ECM molecules, like collagen and matrix-degrading enzymes and inhibitors of metalloproteinases (MMPs), are necessary for the remodeling of basement membranes and the surrounding ECM. ADPKD cyst epithelia are surrounded by a basement membrane with increased expression of matrix components like fibronectin, collagen, MMPs and proteoglycans.<sup>79-82</sup> A recent study showed in zebrafish that a combined knockdown of the *Pkd1* and *Pkd2* resulted in a persistent expression of multiple collagen mRNAs, and low levels of collagen-crosslinking inhibitors, implicating that polycystins are involved in the modulation of

collagen expression and assembly.<sup>83</sup> Furthermore, ECM components, which show increased expression in cystic kidneys, like laminin-5 and periostin, can actively contribute to epithelial cell proliferation and cyst growth, resulting into a positive feedback.<sup>84,85</sup> Cyst-lining epithelia and interstitial fibroblasts are responsible for the production of these structural proteins, enzymes and growth factors. Transforming growth factor  $\beta$  (TGF $\beta$ ), a pro-fibrotic growth factor, is expressed in renal cystic and interstitial cells.<sup>79</sup> In these studies, TGF $\beta$ /Smad2 signaling was increased in PKD mouse models.

### Fluid Secretion

Tubular epithelial cells have the ability to secrete as well as to reabsorb solutes and fluid. Normally, absorptive flux is more than the secretory flux. Sodium chloride reabsorption in collecting duct principal cells is driven by low intracellular sodium concentration generated by basolateral Na-K-ATPase. Sodium chloride enters the apical membrane through the epithelial sodium and chloride channels (Figure 2A). Water enters the cells across the luminal membrane through vasopressin-sensitive aquaporin-2 channels. Cystic epithelial cells are different from normal collecting duct principal cells. Chloride enters across basolateral NaKCC1 cotransporters, driven by the sodium gradient generated by basolateral Na-K-ATPase, and exits across apical protein kinase A (PKA)-stimulated cystic fibrosis transmembrane conductance regulator (CFTR).<sup>86-88</sup> Active accumulation of chloride within the cyst lumen drives sodium and water secretion down transepithelial potential and osmotic gradients (Figure 2B).

**Figure 2. The absorptive and secretory flux in normal collecting duct cells (A) and cystic cells (B).**

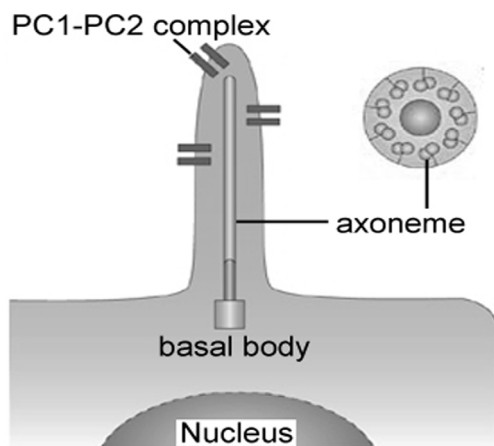


Several lines of evidence suggest an important role of this mechanism in cyst formation. Inhibitors of CFTR and basolateral membrane ion channels retard growth of MDCK cysts in collagen gel, and inhibit 8-Br-cAMP stimulated tubule dilation in metanephric organ cultures.<sup>89,90</sup> In addition, no tubular dilation was observed in metanephric organ cultures from *Cftr*<sup>-/-</sup> mice in response to 8-Br-cAMP.<sup>91</sup> Finally, both CFTR and NKCC1 expression was observed in the cysts of ADPKD patients.<sup>86,88,92</sup> Furthermore, it is shown that *Pkd1* can inhibit CFTR surface expression and activation suggesting that upon decreasing *Pkd1* expression, CFTR surface localization would increase.<sup>93</sup> This model for cyst fluid secretion requires a paracellular pathway sealed by tight junctions impermeable to chloride. If it is not, the transepithelial Cl<sup>-</sup> gradient generated by active secretion would be lost by back-leakage of Cl<sup>-</sup>, reducing the driving force for Na<sup>+</sup> and fluid secretion. Indeed, the composition of tight junctions is altered in cystic epithelia.<sup>94</sup>

### Cilia

It is suggested that PKD arises from abnormalities in the primary cilium.<sup>95</sup> The primary cilium is a hairlike structure that is found on most cells in the body. It consists of a bundle of microtubules, called the axoneme, surrounded by a membrane that is continuous with the cell membrane (Figure 3). The primary cilium is anchored in the cell body by the basal body, which also functions as a centriole during mitosis. Renal epithelial cells have cilia, which project into the tubular lumen and are believed to function as mechano-sensors of urine flow. Fluid flows over the apical surface of the cells, bending the primary cilium, and producing an increase in intracellular calcium concentration, which influence cell signaling pathways.<sup>96</sup> According to this model, PC1 extracellular domain functions as an extracellular sensing antenna on the cilia. PC1 regulates the PC2 ion channel through its intracellular interaction with PC2. Cilia activation induces Ca<sup>2+</sup> influx via PC2 which is further increased through the release of Ca<sup>2+</sup> from the endoplasmic reticulum. Treatment of wild type cells with blocking antibodies against PC2 also inhibits the flow-dependent increase in intracellular Ca<sup>2+</sup> concentration.<sup>34,97</sup> These findings suggest that PC1 and PC2 have a mechanosensory function in renal cilia that is coupled to intracellular Ca<sup>2+</sup> concentration.

**Figure 3. Schematic representation of the primary cilia.**



PKD is believed to be associated with a dysfunction of primary cilia. PC1, PC2, and fibrocystin (protein encoded by the gene mutated in recessive PKD) as well as proteins that are mutated in other cystic kidney diseases, such as nephronophthisis and Bardet-Biedl syndrome, are located in the primary cilium and/or basal body.<sup>98,99</sup> Furthermore, mouse models harbouring mutations involved in cilia formation for example *Ift88* (*Tg737*), *Kif3a* and *Cpk* develop cystic kidneys.<sup>99-102</sup>

Cilia might also regulate orientation of cell division known as planar cell polarity (PCP). A recent study determined PCP in the kidneys of the PKD models, *PCK* rats and *HNF-1 $\beta$*  knockout mice.<sup>103</sup> Using lineage tracing and staining of mitotic cells, it was found that cells in wild type renal tubules divide along an axis that is approximately parallel to the longitudinal axis of the tubule, resulting in tubular elongation without changing the diameter of the tubule. In contrast, in pre-cystic tubules, the orientation of cell division is randomized. This contributes to tubular dilatation, resulting into cyst formation. These results suggest that abnormalities in PCP are present during early stages of cystogenesis. The defects in PCP that are found in PKD may a result of dysfunctional or abnormal primary cilium. Deletion of ciliogenic gene *Kif3a* results in randomized orientation of cell division in pre-cystic tubules that lack primary cilia, indicating aberrant PCP.<sup>55</sup> Similar findings have been observed in mice with collecting duct-specific inactivation of another ciliogenic gene, *Ift20*.<sup>104</sup> These results suggest that abnormalities in primary cilia induce disorientation in PCP that lead to PKD.

### Extra-Renal Cysts

Polycystic liver disease is the most common extrarenal manifestation among ADPKD patients with a frequency of 77% among patients over 60 years.<sup>105</sup> It is likely that in time, somatic mutations and other harmful events accumulate and trigger liver cyst formation.<sup>106</sup> Liver cysts arise by excessive proliferation and dilatation of biliary ductules. Interestingly, PC1 and 2 expression is also detected in the hepatic biliary ductular cells.<sup>42,107,108</sup> Hepatic cysts are more prevalent and have a larger volume in women than in men. Especially in women who have multiple pregnancies or who have used oral contraceptive drugs or oestrogen replacement therapy.<sup>109</sup> Indeed it is reported that estrogen receptors are expressed in the epithelium lining the hepatic cysts, and estrogens stimulate hepatic cyst-derived cell proliferation.<sup>110</sup>

Although polycystic liver disease is asymptomatic, but as the lifespan of patients with polycystic kidney disease has been lengthened with dialysis and transplantation, symptoms have become more common. Symptoms can result from mass effect or from complications related to the cysts, like dyspnoea, early satiety, gastro-oesophageal reflux, mechanical low-back pain, hepatic venous outflow obstruction, compression of the inferior vena cava, portal-vein, or bile-duct. Symptomatic cyst complications include cyst haemorrhage and infection.

Pancreatic cysts are less common in ADPKD patients with a frequency of 9% over 30 years.<sup>111</sup> These patients show only a few cysts in the pancreas which are almost always asymptomatic. These cysts develop from pancreatic tubules, the same region of PC1 and 2 expression.

## Vascular manifestations

Intracranial aneurysms, aortic and cervicocephalic artery dissections (partial rupture of vessel wall) are associated with ADPKD. These patients show a 10-fold higher prevalence of cerebral aneurysms compared to the general population and among ADPKD patients with a family history for aneurysms the prevalence is about 27%.<sup>112,113</sup> For aortic aneurysms, a few clinical studies suggest a prevalence varying from 1-10% in ADPKD.<sup>114-116</sup> Rupture of the vessel wall causes a 35–55% risk of combined severe morbidity and mortality.

Aneurysm is a vascular disorder in which weakness of the artery causes a localized dilatation. This is caused by an imbalanced matrix turnover favoring matrix accumulation and disruption of elastin lamellae, a sheath of highly flexible material providing strength and elasticity to the vessel wall. Aneurysms are usually asymptomatic until rupture occurs, followed by lethal haemorrhage.

PC1 is expressed in vascular smooth muscle cells as well as endothelial cells.<sup>36,42,117</sup> In these cells, PC1 is found at intercellular junctions and cell-matrix junctions. Furthermore, *Pkd1* and *Pkd2* knock-out mouse models die at embryonic days 13.5-14.5 from a primary cardiovascular defect.<sup>36-38,118</sup> These embryos show edema, focal vascular leaks and haemorrhage, suggesting that *Pkd1* and 2 are essential for normal vessel development. Based on these data, it was suggested that PC1 stabilizes adhesion of cells to each other or to the extracellular matrix during and after angiogenesis, thereby maintaining vascular integrity.<sup>37</sup> However, mouse models with reduced expression of *Pkd1* show aneurysms but with an overall normal vessel wall structure, suggesting that a low expression level of PC1 is enough for normal vessel development but not for vessel wall maintenance.<sup>80</sup>

Hypertension is very common in ADPKD, with a prevalence of 50–70% and it is observed before reduction in glomerular filtration rate.<sup>119,120</sup> Furthermore, hypertension occurs at an earlier age in ADPKD patients than in the general population.<sup>121</sup> Renal structural changes in patients with ADPKD have an important role in the pathogenesis of hypertension. Adult patients with ADPKD and hypertension had significantly greater renal volume than patients with normal blood pressure.<sup>122</sup> Immunohistochemical studies of human ADPKD kidneys have shown hyperplasia of renin-secreting cells of the juxtaglomerular apparatus, which suggests chronic stimulation of the Renin-Angiotensin-Aldosterone-System (RAAS) in ADPKD.<sup>123</sup> Furthermore, high levels of renin were found in cyst fluid of ADPKD patients.<sup>124</sup> These findings suggest that activation of the RAAS as a result of cyst expansion and local renal ischemia has an important role in the development of hypertension in this disease.

Besides RAAS, the cilia are also involved in blood pressure regulation. In endothelial primary cilia, PC1 and PC2 are involved in fluid shear sensing and nitric oxide release, thus contributing to vasodilation in response to an increase in blood flow. Aberrant expression of PC2 on cilia could increase blood pressure because of inability to synthesize nitric oxide in response to an increase in shear stress.<sup>125</sup> Furthermore, polycystins are also expressed in arterial smooth muscle cells, which respond to intraluminal pressure that causes wall stretch. An increase in intraluminal pressure causes

myocyte constriction via the stretch-activated cation channels (SACs). Aberrant PC1/PC2 ratio reduces SAC activity and the arterial myogenic tone since PC2 inhibits channel opening, while PC1 reverses this inhibition.<sup>126</sup>

### Aim of Thesis

In this thesis we analyzed the pathogenetic sequence of renal cyst and aneurysm formation in PKD mouse models. In chapter two, we studied cyst formation in *Pkd1<sup>nl/nl</sup>* on the C57Bl6/J background by analyzing tubular cell proliferation, apoptosis, extracellular matrix remodeling, and the expression the Na<sup>+</sup>K<sup>+</sup>2Cl<sup>-</sup> co-transporter (NKCC1). These mice presented with a rapidly progressive phenotype with mild fibrosis and, in time, cyst formation was nephron segment-dependent. Interestingly, the expression of Na<sup>+</sup>K<sup>+</sup>2Cl<sup>-</sup> co-transporters (NKCC1) was also segment-dependent.

In chapter three, we described TGFβ signaling during cysts formation and progression in C57Bl6/J-*Pkd1<sup>nl/nl</sup>* and inducible *Pkd1*-deletion mice.<sup>79</sup> TGFβ signaling is activated at the progressive stages of the disease and coincides with mild fibrosis and increased expression of TGFβ target genes. These results suggest that the TGFβ signaling pathway is probably not implicated in initial steps of cyst formation, but indicate an important role during cyst progression and in fibrogenesis of progressive ADPKD.

In chapter four, we have characterized dissecting aneurysm formation in the hypomorphic mouse model, *Pkd1<sup>nl/nl</sup>* with reduced *Pkd1* transcripts in the kidneys and aorta.<sup>80</sup> Lowering of *Pkd1* transcription levels induces degenerative alterations in both the intima and media of the aorta resulting into a dissecting aneurysm, indicating a direct association between *Pkd1* and vessel wall integrity.

In chapter five, we study the effect of selective disruption of *Pkd1* in vascular smooth muscle cells (SMCs).<sup>127</sup> Remarkably, we did not find any spontaneous gross structural blood vessel abnormalities in mice with somatic *Pkd1* disruption in SMCs or simultaneous disruption of *Pkd1* in SMCs and endothelial cells (ECs). However, cyst formation was detected in pancreas, liver and kidneys. Furthermore, *SM22-Pkd1<sup>del/del</sup>* mice significantly showed reduced decrease in heart rate upon Angiotensin II-induced hypertension. These findings further demonstrate in vivo, that adaptation to hypertension is altered in *SM22-Pkd1<sup>del/del</sup>* mice.

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# Chapter 2

## **The ontogeny of polycystic kidney disease in the hypomorphic C57Bl6/J-*Pkd1*<sup>nl</sup> model**

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## Abstract

Autosomal dominant polycystic kidney disease is characterized by progressive cyst formation in the kidneys. We previously described the hypomorphic *Pkd1*<sup>nl/nl</sup> mouse model that expresses reduced levels of *Pkd1* transcripts. On a mixed 129/Ola;C57Bl6/J genetic background, these mice developed polycystic kidneys with variable severity and lifespan. Here we present the model on C57Bl6/J genetic background. These mice presented with a rapidly progressive phenotype and, in time, cyst formation was nephron segment-dependent, starting with cysts formed in proximal tubules. Interestingly, the expression Na<sup>+</sup>K<sup>+</sup>2Cl<sup>-</sup> co-transporters (NKCC1), which is involved in transepithelial Cl<sup>-</sup> secretion, also demonstrate a segment-dependent pattern. Cystogenesis is accompanied with proliferation but hardly with apoptosis. In time activation of TGFβ signaling increases as well as the expression of genes involved in extra-cellular matrix synthesis and remodeling (*PAI-1*, *Col1*, *EDA-Fn*, *Mmp-2*, *u-Pa*, *Serp1F1*). In parallel, also the mRNA levels encoding potential urinary biomarkers increase (*Lcn2*, *Sgp-2*, *Ptgds*, *C3*). A careful analysis for extra-renal manifestations revealed no manifestations in addition to previously reported dissecting aneurysms. These results indicate that the C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice model seems to reflect the phenotype of patients with early onset PKD as result to two hypomorphic mutations.

## Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is an important cause of end-stage renal disease characterized by bilateral renal cyst formation and a progressive loss of normal kidney tissue that can lead to chronic renal failure. The disease is caused by mutations in *PKD1*, which encodes polycystin-1, and/or *PKD2*, which encodes polycystin-2. These proteins are involved in mechano-sensing, cellular signaling, and cell-cell/cell-matrix interactions.<sup>1-4</sup>

In general, ADPKD patients carry one germ-line mutation and one normal *PKD1* or *PKD2* allele. Renal cysts develop from somatic inactivation of *PKD1* or *PKD2* by a ‘two-hit’ mechanism.<sup>5</sup> In addition, haploinsufficiency, stochastic fluctuations in *PKD1* or *PKD2* gene dosage below a tissue-specific threshold, may suffice to cause cyst formation.<sup>6</sup> Importantly, recent studies indicate that the picture can be more complex, since several families have been reported with non- or incompletely penetrant alleles of *PKD1*.<sup>7,8</sup> At a heterozygous state, they produce no or a very mild phenotype. However, when these hypomorphic alleles are inherited at a homozygous state, or in trans with another mutation, they can produce mild atypical or severe polycystic kidney disease. Evenmore, two hypomorphic *PKD1*-alleles were identified in trans in patients with an ARPKD-like renal cystic phenotype identified in utero, in families with no history of PKD.<sup>8</sup>

We previously described the hypomorphic *Pkd1*<sup>nl/nl</sup> mouse model of polycystic kidney disease that expresses *Pkd1* at a reduced level.<sup>9</sup> *Pkd1*<sup>nl/nl</sup> mice are viable and develop severe PKD, different from mice with homozygous inactivation of *Pkd1* or *Pkd2*, which die in utero due to severe cyst formation in the kidneys and extra-renal defects and/or abnormalities of the placental labyrinth.<sup>10-16</sup> The severity of the phenotype and lifespan varied on a mixed genetic background, 129Ola and C57Bl6/J.<sup>9</sup> In the current study, we studied the ontogeny of cyst formation in *Pkd1*<sup>nl/nl</sup> on C57Bl6/J background, and

analyzed cell proliferation, apoptosis, the expression of the ion co-transporter  $\text{Na}^+\text{K}^+\text{2Cl}^-$  co-transporter (NKCC1) and extracellular matrix remodeling, as well as the expression of genes involved in tissue remodeling and cyst formation. In addition, a careful analysis for extra-renal manifestations revealed no additional manifestations to previously reported dissecting aneurysms.<sup>17</sup>

## Results

### *Pkd1 Expression in C57Bl6/J-Pkd1<sup>nl/nl</sup> Kidneys*

In this study, we analyzed mice bred with C57Bl6/J for 12 generations in order to obtain an almost 100% C57Bl6/J genetic background. Mice were sacrificed at postnatal days 1, 7, 10, 13, 21, and 28. Using reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA), we determined the level of normal and total *Pkd1* transcripts. In general, the level of normal *Pkd1* transcripts was decreased to approximately 20% of the levels in wild type mice (Figure 1A). In wild type mice, *Pkd1* expression decreased as the mice got older, a pattern reflected in the sum of mutant and normal transcripts in the C57Bl6/J-*Pkd1<sup>nl/nl</sup>* mice, only at days 21 and 28, when the kidneys presented a severe cystic phenotype, the expression of the mutant *Pkd1* transcripts was slightly increased, resulting in 1.5-fold higher levels of total *Pkd1* transcripts at day 28 (data not shown). Normal *Pkd1* expression dipped at day 10. The number of mice evaluated at this age, however, was too small to allow for conclusions. Expression of *Pkd2* was not significantly different between mutants and controls, although at 28 days expression was significantly higher compared to 13 days (Figure 1B). Since *Pkd1* and the tuberous sclerosis 2 (*Tsc2*) gene are located, tail-to-tail, only 63 base pairs apart, we analyzed *Tsc2* gene expression.<sup>18</sup> At all ages *Tsc2* gene expression was lower (Figure 1C).

### *Cyst Formation in C57Bl6/J-Pkd1<sup>nl/nl</sup> Kidneys*

Cyst formation was evaluated from postnatal day 1 until the age of 1 month, when the kidneys reached a largely cystic state (Table 1, Figure 2). Nephron segment-specific markers were used to identify the origin of renal cysts. Antibodies against megalin were used to stain the proximal tubules,<sup>19</sup> antibodies against uromodulin were used to label the thick ascending limb of Henle and distal convoluted tubules<sup>20</sup> and antibodies against anti-aquaporin-2 identified collecting ducts.<sup>21</sup>

In newborn mice, at days 1 and 3, many dilated proximal tubules were present in the inner cortex (Figure 2, A and C, and Figure 3A). At day 7, proximal cysts were found in the cortico-medullary region between the mesenchymal cells (Figure 2E and Figure 3B). A few of these cysts exhibited a partial loss of megalin staining (Figure 3B, arrow). The nephrogenic zone was normal.<sup>22</sup> In the medulla, many small cysts and dilatations started to develop in the collecting ducts at day 7 (Figure 3C). The cysts and dilatations were derived from distal tubules or collecting ducts. A few dilatations were negative for all markers used. From day 10 onward, the epithelial cysts occupied the entire cortex and medulla, and they increased in number and size until the age of 3 and 4 weeks (Figure 2, G, I, K, and M). At days 10 and 13, the majority of cysts in the cortex and medulla were derived from the collecting ducts (Figure 3D). Some dilated distal tubules were present at day 13. At days 21 and 28, most cysts were positive for distal tubule or collecting duct epithelial markers (Figure 3E).

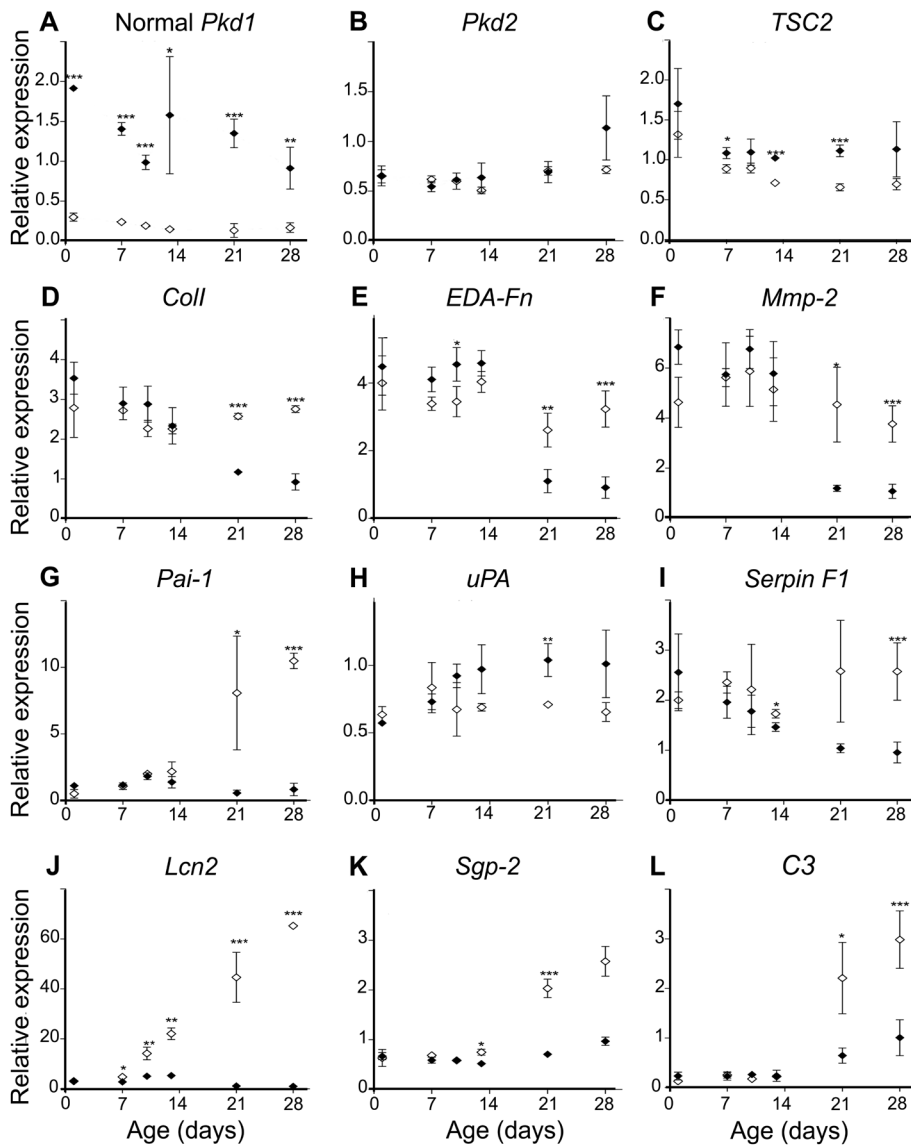


**Figure1. Relative mRNA transcription levels of *Pkd1*, *Pkd2*, *Tsc2*, SMAD2-target genes and biomarkers genes in kidneys from wild type and C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice.** The expression of each gene versus reference gene *Hprt* has been normalized to the mean of wild type mice at day 28, which has been set to 1. The expression levels of the wild type and mutant mice of the same age were compared with an unpaired 2-tailed t-test. (A) The 5' *Pkd1* PCR amplifies normal *Pkd1* transcripts. The expression level of normal *Pkd1* transcription in the kidney of *Pkd1*<sup>nl/nl</sup> mice shows a reduction to approximately 20% compared to wild type. (B) Expression of *Pkd2* was not significantly different between mutants and controls. (C) At all ages *Tsc2* gene expression was lower in mutant kidneys compared to wild types. (D-I) At early neonatal stages expression of all SMAD2-target genes is not significantly different compared to wild types. At days 21 and 28, however, transcript levels are increased in mutants compared to wild types.(D) Collagen1 (*Coll*). (E) EDA-

fibronectin (*EDA-Fn*). (F) Matrix metalloproteinase-2 (*Mmp-2*). (G) Plasminogen activator inhibitor 1 (*Pai-1*). (H) Urokinase-type plasminogen activator (*uPA*) is reduced compared to wild type starting from day 10. (I) Expression of another inhibitor in plasmin synthesis, alpha-2 antiplasmin (*Serpin F1*), is increased. (J-L) Three biomarkers which monitor disease progression were analyzed. The expression levels of these genes were increased with disease progression. (J) Neutrophil gelatinase-associated lipocalin (*Lcn2*). (K) Sulfated glycoprotein-2 (*Sgp-2*). (L) Complement C3 (*C3*). Black diamonds: wild type; white diamonds: C57Bl6/J-*Pkd1*<sup>nl/nl</sup> \*:p=0.01-0.05; \*\*: p=0.01-0.001; \*\*\*p<0.001

**Table 1. Cyst formation in the C57Bl6/J-*Pkd1*<sup>nl/nl</sup> from day 1 until day 28.**

| Age        | Renal Pathology                                                                                                                                                                                                                                                                                                                                  |
|------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Day 1-3    | <ul style="list-style-type: none"> <li>• Kidneys not fully developed</li> <li>• Proximal dilated tubules in inner-cortex.</li> <li>• Proliferation not increased and no indication of apoptosis</li> </ul>                                                                                                                                       |
| Day 7      | <ul style="list-style-type: none"> <li>• Large fusiform proximal cysts in cortico-medullary junction</li> <li>• Numerous small cysts in medulla from collecting duct origin</li> <li>• Proliferation not increased and no indication of apoptosis</li> </ul>                                                                                     |
| Day 10-13  | <ul style="list-style-type: none"> <li>• Entire kidney occupied by cysts</li> <li>• Most cysts originate from collecting ducts and few cysts originate from distal tubules.</li> <li>• No glomerular cysts</li> <li>• Proliferation not increased and no indication of apoptosis</li> <li>• Accumulation of collagen around few cysts</li> </ul> |
| Day 21 -28 | <ul style="list-style-type: none"> <li>• Progressive cystic kidneys with many large cysts and a few normal tubules.</li> <li>• Proliferation increased in tubules and cysts at the cortico-medullary junction at day 21</li> <li>• Apoptosis in large cysts.</li> <li>• Accumulation of collagen around many cysts</li> </ul>                    |

Furthermore, some cysts stained for both of these markers. A few cysts were positive for megalin, whereas other cysts did not stain for any epithelial marker. The latter group of cysts may be proximal cysts that have lost megalin expression, as previously reported for the *Cy/+* rat.<sup>23</sup> Some cysts were fusiform in shape (Figure 3D, arrow); however, the most inner part of the papilla exhibited relatively normal renal tissue with a few dilated collecting ducts. Glomerular cysts were not found at any age. We did not observe any gender differences.

*Extra-renal Manifestations*

In order to determine extra-renal abnormalities, we analyzed the pancreas and livers of three C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice aged one month and did not observe cysts in these organs. We did not observe any ductal plate malformations or portal fibrosis. We previously reported dissecting aneurysms in the aorta and mesenteric artery of these mice.<sup>24</sup>

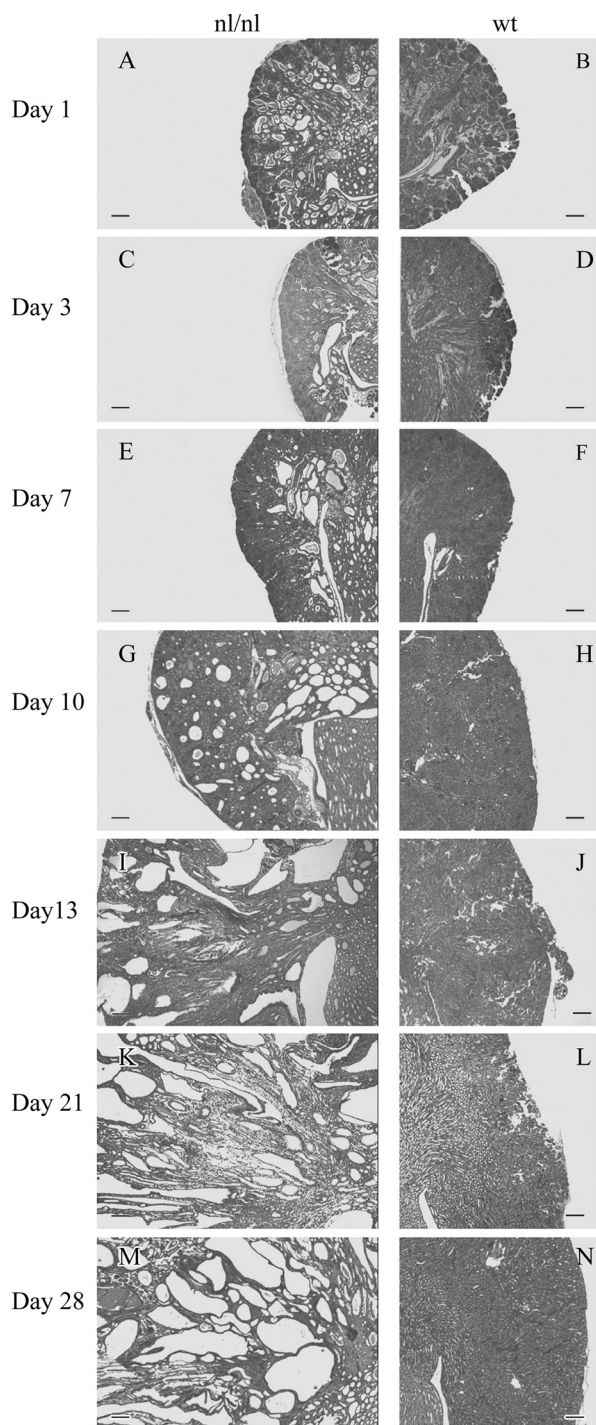


**Figure 2. Time course of development of polycystic kidney disease in C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice from day 1 until day 28.**

At young ages (day 1-7) dilated tubules and a few cysts can be observed, while at older ages, from day 10 on, the entire kidney is occupied with cysts. At days 21 and 28, the kidneys reach a very progressive stage with loss of normal renal tissue. A, C, E, G, I, K, M: C57Bl6/J-*Pkd1*<sup>nl/nl</sup> kidneys, and B, D, F, H, J, L, N: wild type kidneys. Haematoxylin Eosin staining. Bar=100µm

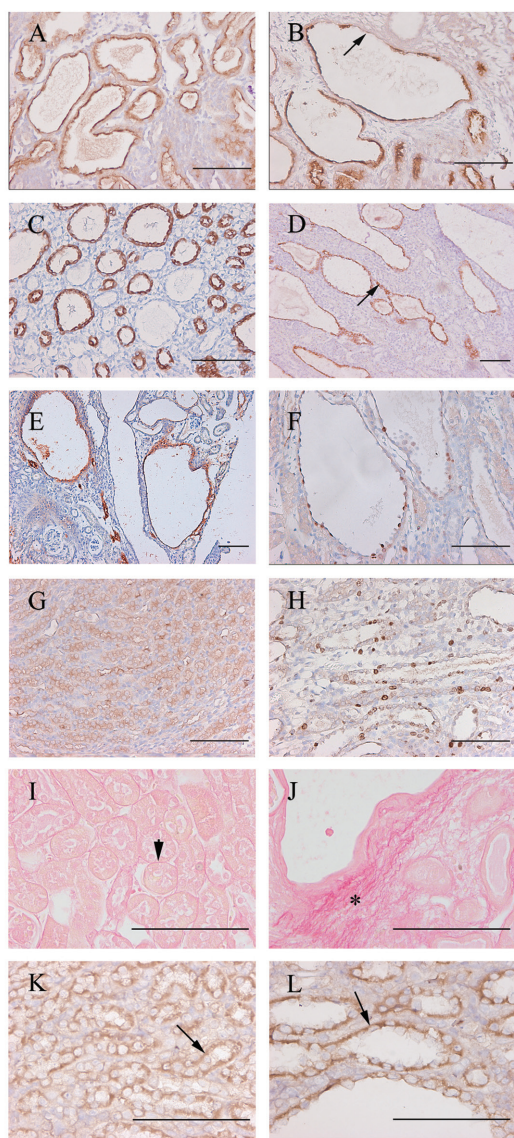


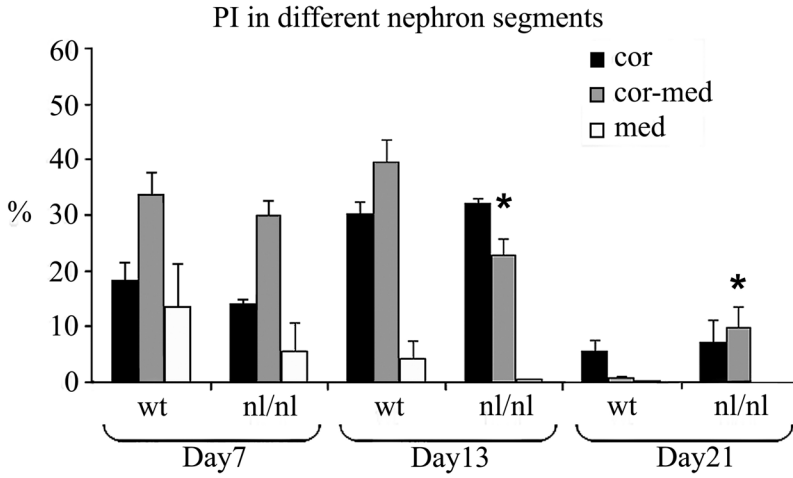
**Figure 3. Immunohistochemical Analysis of renal cysts in C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice.**

(A) 3-days-old kidney shows dilated tubules in the cortex, which stain for megalin. (B) At day 7, the small cysts in this region show focal expression of megalin (B arrow). (C) At day 7 dilated tubules and small cysts start to develop in the medulla. Most of them express aquaporin-2. (D) At day 13 the majority of cysts in cortex and medulla show expression of aquaporin-2. Some of these cysts are fusiform (arrow D). (E) Cysts originating from distal tubules, expressing uromodulin, are at 21. (F) Cysts showing many proliferating cells at day 21. The region of this cyst is the cortex. (G) In the cortico-medullary region of wild type kidney almost no proliferating cells are present, day 21. (H) However, dilated tubules in the same region of the mutant kidney about 10% of the cells are mitotic, day 21. (I) Collagen, stained by Sirius red, in a wild type 4-weeks-old kidney. The collagen fibers are concentrated around the tubules (arrowhead). (J) Collagen in a C57Bl6/J-*Pkd1*<sup>nl/nl</sup> 4-weeks-old-kidney. Interstitial collagen accumulation is found around cysts (asterisk). (K) The 21-days-old wild type kidney shows basolateral localization of NKCC1 in the outer medulla (arrow). (L) The same basolateral pattern for NKCC1 is found in tubules and cysts of C57Bl6/J-*Pkd1*<sup>nl/nl</sup> kidneys at the same region and age (arrow). A, B: megalin, C, D: aquaporin-2, E: Uromodulin, F-H: Ki67, I-J: Sirius red. K-L: NKCC1. Bar=100µm.

### *Proliferation, Apoptosis, and Extracellular Matrix Accumulation in C57Bl6/J-Pkd1<sup>nl/nl</sup> Kidneys*

A variety of studies suggest that increased proliferation and/or apoptosis is essential for cyst formation.<sup>25-28</sup> Antibodies against Ki67, which stains mitotic cells, and antibodies against active caspase-3, which detects the terminal phase of apoptosis, were used. The proliferation index (PI) was determined at days 7, 13, and 21 (Figure 4). Proliferation was highest in tubules and small cysts in the cortex and cortico-medullary region of both

A



B

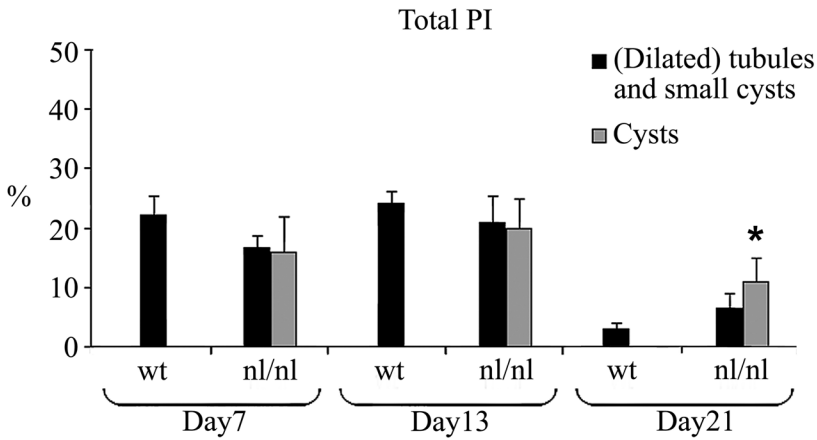


**Figure 4. Proliferation index (PI) in wild type and C57Bl6/J-*Pkd1*<sup>nl/nl</sup> kidneys.**

(A) PI values in different nephron segments of (dilated) tubules and small cysts in mutant kidneys compared to wild type kidneys. Cortico-medullary regions at days 13 and 21 in mutants show a significantly different PI compared to wild type kidneys (at day13 wild type mean  $39.58 \pm 3.91$  versus mutant mean  $22.92 \pm 2.94$   $p=0.04$ ; at day 21 wild type mean  $0.68 \pm 0.48$  versus mutant mean  $9.73 \pm 3.81$   $p=0.049$ ). (B) PI values in (dilated) tubules and small cysts compared to larger cysts in wild type and mutant kidneys. At day 7, dilated tubules and cysts in mutants show a slightly lower PI compared to total wild type tubules. The proportion of mitotic cells in cysts is significantly higher than in cells of wild type kidneys at day 21 (mean  $10.88 \pm 4.08$  versus mean  $3.06 \pm 0.85$   $p=0.03$ ). \*= $p<0,05$

wild type and mutant kidneys (Figure 4A). In wild type and mutant mice, proliferation was highest in the early developmental stages, days 7 and 13. At day 21, the percentage of proliferating cells strongly decreased in wild type mice, but not as much as in the mutants (wild type  $0.68 \pm 0.48$  versus mutant  $9.73 \pm 3.81$ ,  $P=0.05$ ) (Figure 3, G and H, and Figure 4A). Furthermore, at day 21, cysts exhibited more proliferating cells than the nephrons in wild type kidneys (Figure 3F and Figure 4B). The PI, however, was not different between small and large cysts. We observed increased proliferation in some groups of cysts and dilated tubules, indicating that proliferation may occur in regional bursts rather than at random.

In general, Apoptosis was minimal, and it was comparable in wild type and mutant kidneys at days 7-21. Only at the ages of 3 and 4 weeks a few apoptotic cells were observed in the epithelium of large cysts, indicating that apoptosis may not be a major factor for early cystogenesis in this mouse model.

Collagen accumulation was analyzed by Sirius red staining. At early ages, no signs of collagen deposition were observed, but, at days 10 and 13, collagen started to accumulate around some cysts. The area of collagen deposition and the number of cysts surrounded by collagen deposition increased at days 21 and 28 (Figure 3, I through J). Collagen is a target of SMAD2, a transcription factor involved in TGF $\beta$  signaling. In cystic kidneys of several *Pkd1*-mutant mouse models transcript levels of SMAD2 target genes are up-regulated, e.g. matrix metalloproteinase-2 (*Mmp-2*), plasminogen activator inhibitor 1 (*Pai-1*), collagen type 1 (*Col1*) and EDA-fibronectin (*EDA-Fn*).<sup>29</sup> At early neonatal stages (days 1, 7, 13) expression of all these genes, which are involved in extracellular matrix synthesis and remodeling, is not significantly different compared to wild types (Figure 1D-I). At days 21 and 28, however, when renal development is completed, transcript levels decrease in wild types while in mutants expression levels are less decreased or are (strongly) increased. At all stages, transcript levels of *Mmp14*, encoding the activator of *MMP2*, show a very similar expression pattern as for *Mmp2* (data not shown). For other genes involved in extracellular matrix remodeling, expression of the plasminogen activator inhibitor 1 (*Pai-1*) is strongly up-regulated in cystic kidneys, while expression of its target urokinase-type plasminogen activator (*u-PA*) is reduced (Figure 1G-H). Expression of another inhibitor in plasmin synthesis, alpha-2 antiplasmin (*Serpin F1*), is increased as well (Figure 1I).<sup>30</sup>

Expression of genes encoding biomarkers Neutrophil gelatinase-associated lipocalin (*Lcn2*), sulfated glycoprotein-2 (*Sgp-2*) or clusterin and prostaglandin D2 synthase (*Ptgds*) are potential biomarkers to monitor disease progression. For all three genes, expression levels increase with disease progression (Figure 1J-K). Also complement C3 (*C3*), a potential biomarker and is an important player in the innate immunity response, is highly elevated in cystic kidneys (Figure 1L).

#### *NKCC1 expression during Cyst Formation in C57Bl6/J-Pkd1<sup>nl/nl</sup> mice*

Transepithelial Cl<sup>-</sup> secretion involves two steps: basolateral NKCC1 concentrates Cl<sup>-</sup> in the cell and apical CFTR channels release Cl<sup>-</sup> into the cyst lumen.<sup>31</sup> It has been reported that NKCC1 localizes at the basolateral side of the cell.<sup>32</sup> In C57Bl6/J-*Pkd1<sup>nl/nl</sup>* mice, a clear basolaterale staining was not always detected due to diffuse cytoplasmic staining.

The expression NKCC1 demonstrate a segment-dependent pattern. In proximal tubules and proximal cysts, NKCC1 was clearly expressed in the cytoplasm on days 1 through 7. On days 10 and 13, weak expression was observed in the medulla and in cysts originating from the collecting ducts and distal tubules. On days 21 and 28, the outer medulla exhibited slightly more staining for NKCC1 than in the inner medulla (Figure 3, K and L) (Table 2).

**Tabel 2. NKCC1 expression in C57Bl6/J-*Pkd1*<sup>nl/nl</sup>**

| Age         | NKCC1 expression in C57Bl6/J- <i>Pkd1</i> <sup>nl/nl</sup>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
|-------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Days 1-3    | <ul style="list-style-type: none"> <li>• Cytoplasmic NKCC1 in (dilated) proximal tubules and proximal cysts.</li> <li>• Wild type kidneys show cytoplasmic NKCC1 in proximal tubules.</li> <li>• Weak NKCC1 expression in (dilated) tubules in medulla.</li> <li>• Wild type kidneys show weak cytoplasmic NKCC1 in the medulla.</li> </ul>                                                                                                                                                                                                                             |
| Day 7       | <ul style="list-style-type: none"> <li>• Cytoplasmic NKCC1 in (dilated) proximal tubules and proximal cysts.</li> <li>• Wild type kidneys show cytoplasmic NKCC1 in proximal tubules.</li> <li>• Weak NKCC1 expression in (dilated) tubules in medulla.</li> <li>• Wild type kidneys show weak cytoplasmic NKCC1 in the medulla.</li> </ul>                                                                                                                                                                                                                             |
| Days 10-13  | <ul style="list-style-type: none"> <li>• Cytoplasmic NKCC1 in proximal tubules of mutant kidneys.</li> <li>• Wild type kidneys show cytoplasmic NKCC1 in proximal tubules.</li> <li>• Weak cytoplasmic NKCC1 expression in the medulla, in distal tubules, collecting ducts and in cysts in mutants.</li> <li>• Wild type kidneys show weak cytoplasmic NKCC1 in the medulla.</li> </ul>                                                                                                                                                                                |
| Days 21 -28 | <ul style="list-style-type: none"> <li>• Cytoplasmic NKCC1 expression in proximal tubules of mutant kidneys.</li> <li>• Wild type kidneys show cytoplasmic NKCC1 in proximal tubules.</li> <li>• Weak cytoplasmic NKCC1 expression in the medulla, in distal tubules, collecting ducts and in cysts in mutants. Only small cysts and tubules in the outer medulla show basolateral NKCC1 expression, like tubules of the same region in wild type kidney.</li> <li>• The outer medulla exhibited slightly more staining for NKCC1 than in the inner medulla.</li> </ul> |

**Discussion**

The *Pkd1*<sup>nl/nl</sup> mouse model is an orthologous model for polycystic kidney disease. The severity of the phenotype varies among mice with a mixed genetic background.<sup>9</sup> In this study, we found that, on a C57Bl6/J background, these mice present with a rapidly progressive phenotype and die between 3 and 4 weeks of age. All models homozygous for a *Pkd1* inactivating allele present with severe cystic kidneys around E15, indicating that polycystin-1 is required for normal kidney development.<sup>10-13,16</sup> The C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice, however, express low levels of *Pkd1*, are viable, and exhibit grossly normal kidney structure but with dilated proximal tubules at birth.

The first cysts develop from the fully mature proximal tubules and not from the comma or S-shaped bodies in the nephrogenic zone in the outer cortex. This is followed by a phase of distal tubule and collecting duct cyst formation and progressive enlargement leading to renal failure. In contrast to knock-out mice, C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice do not develop glomerular cysts, indicating that the low expression of *Pkd1* is sufficient to maintain a normal glomerular structure, and the total loss of *Pkd1* induces glomerular cysts during embryonic development.<sup>11,12,16,33</sup> Those glomerular cysts observed in *Pkd1* knock-out mice were only observed for glomeruli located in the deep medulla zone, whereas superficial glomeruli located in the cortex among comma and S-shaped bodies were non-cystic.<sup>33</sup>

Interestingly, in a recent study two pedigrees were described without family history of polycystic kidney disease, each containing two patients with in utero onset of PKD. The renal cystic phenotype was typical for ARPKD. Mutation analysis, however, did not reveal mutations in the *PKHD1* gene, but revealed two hypomorphic *PKD1* alleles.<sup>8</sup> Histologic analysis of renal tissue of an affected fetus obtained after abortion at 20 weeks pregnancy revealed that the majority of cysts (77%) were of proximal tubular origin. Thus, in both the mouse model as well as the patient carrying two hypomorphic *PKD1* alleles, the first cysts are formed in the proximal tubules.

The pattern of nephron segment-specific cyst formation during nephrogenesis could be a reflection of *Pkd1* expression. The first cysts are formed in the proximal tubules, but, over time, cysts derived from the collecting ducts and distal tubular segments can be identified. In immature proximal tubules, *Pkd1* expression is high and declines progressively in mature proximal tubules but starts to increase in the loop of Henle, distal tubules, and collecting ducts, a pattern that persists in adult kidneys.<sup>34,35</sup> Also, in other models of ADPKD or ARPKD (e.g. *cpk/cpk*, *BALB/c-bpk/bpk*, *TG737orpk*, and *kat2J* mutant mice, and *Han:SPRD* and *wpk* rat models), this segment-specific pattern of cyst formation has been observed.<sup>36-44</sup>

Since *Pkd1* and the tuberous sclerosis 2 (*Tsc2*) gene are located, tail-to-tail, only 63 basepairs apart we analyzed *Tsc2* gene expression.<sup>18</sup> At all ages *Tsc2* gene expression was lower. In mice with neonatally induced *Pkd1* inactivation *Tsc2* expression was also reduced at 28 days. However, in mice with *Pkd1* inactivation at adult age, expression was not altered (data not shown), suggesting that the combination of developmental stage and *Pkd1* inactivation affects gene expression.

A variety of studies indicate increased apoptosis and/or cell proliferation in the cystic kidneys of animal models and ADPKD patients.<sup>25-28</sup> In C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice, the overall proliferation index in the renal epithelium at neonatal stages was not significantly higher compared to wild type mice, which is similar to what has been reported for young inducible *Pkd1* deletion mice.<sup>33,45</sup> Proliferation is much higher in the kidneys of 7- and 13 day-old mice compared to 21 day-old mice, a phenomenon related to a developmental switch in kidney maturation.<sup>45</sup> Comparing epithelial cell proliferation in cortex, cortico-medullary region and medulla, proliferation is at days 7 and 13 highest in the cortico-medullary region, both in wild type and mutant kidneys. This is a major growth zone for medullary structures and the region from which the majority of cysts are derived in

C57Bl6/J-*Pkd1*<sup>nl/nl</sup>.<sup>46</sup> This is in line with our previous report showing that knocking-out *Pkd1* at postnatal day 4 results in massive cyst formation with the major involvement of the distal nephron, whereas gene disruption in adults (5.5 weeks and 3 to 5 months of age) results in cyst formation in all segments of the nephron.<sup>33,47</sup>

A driving force for transepithelial fluid secretion is transepithelial Cl<sup>-</sup> secretion mediated by NKCC1 and CFTR.<sup>31</sup> In general, the nephron-segment expression pattern of NKCC1 in cystic kidneys is similar to the pattern observed in wild type kidneys, e.i. relatively strong expression was observed in proximal tubule derived cysts, but only weak expression in cysts derived from distal tubules and collecting ducts. In C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice, the first cysts are formed in the proximal tubules. Accordingly, in this model, the strongest NKCC1 expression is observed in cysts at younger ages. In different inducible *Pkd1* deletion mice, only weak expression of NKCC1 was observed in mice with cysts derived from the distal part of the nephron, while high expression was seen in a model with a large proportion of cysts being of proximal tubular origin (not shown).

In C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice, the accumulation of collagen is relatively mild compared to human cystic kidneys, probably because these mice do not live long enough for the development of progressive fibrosis. Expression of SMAD2 targets (*Mmp2*, *EDA-Fn*, *Col1* and *Pai-1*) all show a developmental expression pattern, e.g. expression is 2 to 5 times down-regulated in wild types at days 21 and 28, compared to days 1-13 (Figure 1 D-H). This is in agreement with reduced expression of TGFβ receptors in adult rats compared to developmental stages.<sup>48</sup> In cystic kidneys, however, expression is moderate to strongly up-regulated compared to wild type littermates. Plasminogen activator inhibitor 1 (*Pai-1*) is strongly up-regulated in cystic kidneys, while expression of its target urokinase-type plasminogen activator (*uPA*) is reduced. Expression of another inhibitor in plasmin synthesis, alpha-2 antiplasmin (*SerpinF1*), is increased as well (Figure 1I). Plasmin is a serine protease with a broad substrate specificity including the extracellular matrix proteins laminin and fibronectin, either directly or indirectly via MMP's.<sup>30</sup> Although the role of the plasminogen activator system in PKD is not clear yet, these data suggest a role in extracellular matrix accumulation via reduced degradation of ECM components.

Urinary biomarkers reflecting renal tubular damage or inflammation are getting increasing attention.<sup>49</sup> We measured increased expression of transcripts encoding *LCN2*, sulfated glycoprotein-2 (or clusterin), prostaglandin D2 synthase and complement C3 in cystic kidneys (Figure 1J-L). These genes were selected because the encoded proteins are potential biomarkers and/or differential expression observed expression arrays. The latter three proteins were also measured in a proteomic analysis of cyst fluid.<sup>50</sup> An association of urinary levels of *LCN2* and renal volume has previously been reported for ADPKD<sup>49</sup> and elevated expression of sulfated glycoprotein-2 and complement C3 has previously been reported in renal cystic tissues.<sup>51</sup>

The observed early mortality is probably caused by rupture of dissecting aneurysms, which are found in large vessels like the aorta and mesenteric artery at the age of 3 weeks in most C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice (8 out of 9),<sup>24</sup> although renal failure cannot be entirely excluded because blood urea levels were moderately elevated in a few mice,

at 3 weeks (data not shown). These manifestations may also occur more frequently or more severe in the patients with two hypomorphic alleles. On the other hand, in general patients with ADPKD show variation in disease progression, and although the factors that affect this diversity are not clear, modifying genes and environmental factors are probably involved.<sup>52</sup> Other extra-renal manifestations that can also be (less frequently) found in ADPKD patients are cysts in liver and pancreas, but were not observed in these mice.<sup>9,17,53</sup>

There are phenotypic differences between ARPKD and ADPKD. Ductal plate malformations and congenital hepatic fibrosis are considered characteristic for ARPKD. As in C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice, also the patient with two hypomorphic *Pkd1* alleles who died in utero with severe renal cystic disease, did not show these manifestations.<sup>8</sup> Renal cysts, however, had a fusiform appearance as in ARPKD, probably because at the same time many epithelial cells become cysts, rather than a single cell or small group of cells as result of second hits.

In conclusion, the renal cystic phenotype of C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice, seems to reflect the phenotype of patients with early onset PKD as result to two hypomorphic mutations. The first cysts are formed in the proximal tubules, but, over time, cysts derived from the collecting ducts and distal tubular segments can be identified.

## Methods

### Mice

The *Pkd1*<sup>nl/nl</sup> mice were generated as previously described in detail.<sup>9</sup> A loxP-flanked neomycin selection cassette was inserted into intron 1 of *Pkd1* by homologous recombination and a third loxP site was inserted into intron 11. Chimeric offspring were backcrossed with C57Bl6/J mice for 12 generations, resulting in an almost 100% C57Bl6/J background.

All experiments with mice were approved by the Animal Care and Use Committee of Leiden University and by the Commission for Biotechnology in Animals of the Dutch Ministry of Agriculture.

### Immunostaining

Immunohistological analysis was performed on 24 *Pkd1*<sup>nl/nl</sup> and 22 wild type kidneys from mice of different ages (1, 3, 7, 10, 13, 21, and 28 days old). The kidneys were fixed in 4% phosphate buffered formalin, dehydrated in graded ethanol and xylene, and embedded in paraffin. Sequential sections of 4 µm were mounted onto superfrost-plus glass slides. After de-paraffinization, sections were stained using standard procedures.<sup>54</sup> Antigen-retrieval, at 100°C for 10 min in citric acid buffer (0.01 M, pH 6.0), was performed for Ki67 and caspase-3; and it was performed for NKCC1 in Tris:EDTA (10:1 mM). The antibody complexes were visualized using 0.04% diaminobenzidine-tetra-hydrochloride (DAB) with 0.5% CuSO<sub>4</sub> or 0.7% NiCl<sub>2</sub>. Incubation of the NKCC1 antibody with a 50-fold excess of blocking peptide completely eliminated staining. For histological analysis kidney sections were stained with hematoxylin-eosin (HE) and Sirius red (collagen staining). Sirius Red stains collagen type I, II and III.<sup>55,56</sup>

### Antibodies

The following primary antibodies were used: anti-caspase-3 (1:300; Cell Signaling, Leiden, the Netherlands), anti-Ki67 (1:3000; Novocastra, Valkenswaard, the Netherlands), anti-megalin (1:500; Pathology, Leiden, the Netherlands),<sup>19</sup> anti-uromodulin/Tamm Horsfall (1:3000; CAPEL, Durham, NC), anti-aquaporin-2 (1:300; Calbiochem, Amsterdam, the Netherlands), anti-NKCC1 (1:100; Santa Cruz, Heidelberg, Germany). Secondary antibodies were rabbit envisions/HRP (undiluted; Dako, Heverlee, Belgium) and rabbit-anti-goat Ig/HRP (1:100; Dako, Heverlee, Belgium).

### mRNA Quantifications

Transcription levels were determined by Reverse Transcriptase Multiplex Ligation-dependent Probe Amplification (RT-MLPA). The reactions were performed as previously described.<sup>57</sup> Briefly, 21 wild type and 19 *Pkd1*<sup>nl/nl</sup> kidneys from mice of different ages (1, 7, 10, 13, 21, and 28 days old) were homogenized and total RNA isolated. From an aliquot of 200-300 ng total RNA, cDNA was synthesized. Two separate hybridization

reactions were performed with this cDNA. To each sample, MLPA probe mix and SALSA MLPA buffer were added (MRC-Holland, Amsterdam, the Netherlands), and the samples were incubated at 95°C for 1 min followed by 60°C for 4 hours. We developed two *Pkd1* probe sets: the 5' *Pkd1* probe set specifically identified the normal *Pkd1* transcript and the 3' *Pkd1* probe set identified both mutant and normal *Pkd1* transcripts. The sequences of probes and oligonucleotides are available upon request. The ligation of the annealed oligonucleotides was performed at 54°C for 10-15 min. The ligase enzyme was inactivated by heating at 98°C for 5 min, and the ligation products were amplified by PCR using SalsaTaq (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands). The amplified samples were mixed with Hi-Di Formamide containing GeneScan-500 ROX size standard (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands) heated for 5 min at 95°C and run on a 3730 DNA analyzer (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands). Data were analyzed using GeneScan Analysis 3.5 and Microsoft Excel software. For situations in which off-scale signals needed to be reduced, competitor oligonucleotides were applied. The expression of the housekeeping gene *Hprt* (hypoxanthine guanine phosphoribosyl transferase) served as references for gene expression. Peak ratios of the target gene and housekeeping gene were calculated, and results of the mutant and wild type kidneys were normalized to the mean of wild type kidneys at day 28, which was set to 1. The expression levels of the wild type and mutant mice of the same age were compared with an unpaired 2-tailed t-test. A p-value of <0.05 (2-tailed) was considered significant.

#### *Proliferation Index (PI)*

The PI was calculated for 11 wild type and 12 mutant kidneys from mice of different ages (7, 13, and 21 days old) as previously described by counting the percentage of Ki-67-positive renal epithelial cells in four to ten randomly selected fields of a cross-section.<sup>33</sup> Between 1000 and 3000 tubular epithelial nuclei of normal, dilated tubules and small cysts were counted per mouse. From large cysts, which were defined as being five times larger than normal tubules, between 300 and 1000 nuclei were counted per mouse. Only clearly definable heavy or granular nuclear staining was identified as positive. The PI values of the wild type and mutant mice were compared with an independent sample 2-tailed t-test. The PI values of (dilated) tubules and large cysts within one mouse were compared by a paired 2-tailed t-test. A p-value of <0.05 (2-tailed) was considered significant.

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# Chapter 3

## **Elevated TGF $\beta$ /Smad signaling in experimental *Pkd1* models and human patients with polycystic kidney disease**

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## Abstract

Autosomal dominant polycystic kidney disease (ADPKD) is a common inherited renal disease characterized by many fluid-filled cysts and interstitial fibrosis in the kidneys leading to chronic renal failure. During cystogenesis the renal tubules undergo extensive structural alterations that are accompanied by altered cellular signaling, directly and/or indirectly regulated by the *PKD1* and *PKD2* proteins.

Since transforming growth factor  $\beta$  (TGF $\beta$ ) signaling modulates cell proliferation, differentiation, apoptosis, adhesion, and migration of various cell types, we studied the activation of this signaling pathway in *Pkd1*-mutant mouse models at different stages of the disease. Therefore, we analyzed expression the TGF $\beta$ /Smad signaling pathway and its target genes in different *Pkd1* mutant mouse models in various stages of polycystic disease. Nuclear accumulation of P-Smad2 in cyst lining epithelial cells was not observed in the initiation phase but was observed at mild and more advanced stages of PKD. This coincides with mild fibrosis and increased mRNA-levels of TGF $\beta$  target genes, like fibronectin, collagen type I, plasminogen activator inhibitor 1 and matrix metallo-proteinase-2. At this stage many interstitial fibroblasts were found around cysts, which also showed nuclear localization for P-Smad2. However, bone morphogenetic protein (BMP) signaling, which can antagonize TGF $\beta$  signaling, is not affected since nuclear expression of P-Smad1/5/8 and expression of the BMP target gene, inhibitor of DNA binding/differential-1 (*Id-1*) is not altered compared to wild type controls. Also human kidneys with progressive ADPKD showed increased nuclear localization of P-Smad2 while in general expression of P-Smad1/5/8 was weak. These results exclude TGF $\beta$  signaling at the initiation of cystogenesis, but indicate an important role during cyst progression and in fibrogenesis of progressive ADPKD.

## Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common inherited diseases. In 85% of the patients, the gene responsible for the disease is *PKD1*, which encodes polycystin-1, whereas in 15% of patients the mutation is in the *PKD2* gene, which encodes polycystin-2. The proteins are involved in a variety of functions like mechano-sensing, cellular signaling and cell-cell/cell-matrix interactions.<sup>1-4</sup> The main features of the disease are the formation of many fluid-filled cysts in the kidneys as well as interstitial fibrosis leading to progressive loss of normal renal tissue and resulting in chronic renal failure.<sup>5</sup> Cystogenesis is characterized by proliferation of the epithelium, excessive secretion of solute and fluid into the cysts and remodeling of the extracellular matrix, processes that are also regulated by growth factors, hormones, and cytokines of the kidneys.<sup>1</sup>

The transforming growth factor  $\beta$  (TGF $\beta$ ) super family members are multifunctional cytokines that modulate many of these process like proliferation, differentiation, apoptosis, adhesion, cell migration and these cytokines are believed to play a crucial role in fibrotic deposition.<sup>6</sup> Indeed, several lines of evidence suggest that altered TGF $\beta$  signaling is implicated in PKD. Mice with overexpression of the BMPs receptor activin receptor-like kinase (ALK)-3 as well as *Bmp-7* knock-out mice develop polycystic kidneys.<sup>7,8</sup> Also BMP-7 treatment of the *jck* mouse, retarded cyst formation.<sup>9</sup> In addition, increased levels of TGF $\beta$  transcripts are found in kidneys of the *pcy* model and reduced expression of P-Smad1 has been reported for the *cpk/cpk* mouse.<sup>10,11</sup> However, none of

these PKD models have a targeted mutation in the *Pkd1* or 2 genes. In addition, several studies performed on end-stage ADPKD-kidneys suggested involvement of TGF $\beta$  signaling in polycystic kidney disease.<sup>12-14</sup> In the current study, we analyzed the activation of the TGF $\beta$ /Smad signaling pathway at different stages of polycystic disease in different *Pkd1* mutant mouse models and in human ADPKD-tissues.<sup>15,16</sup>

## Results

In this study we analyzed TGF $\beta$  and BMP signaling in different *Pkd1* mouse models at different stages of disease. These cytokines bind to a pair of serine/threonine kinase transmembrane receptors that mediate the phosphorylation of receptor-regulated Smads (R-Smads). R-Smad2 and 3 are activated by TGF $\beta$ , and R-Smads1, 5 and 8 act downstream of BMP. Phosphorylated R-Smads can form a heteromeric complex with Smad4 and these complexes accumulate in the nucleus where they act as transcription factors.<sup>6</sup>

### *TGF- $\beta$ and P-Smad2 Expression at Different Stages of PKD in C57Bl6/J-Pkd1<sup>nl/nl</sup> Kidneys*

C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice have reduced levels of *Pkd1* gene expression and present a rapidly progressive phenotype. The first dilated tubules can be seen at day 1 and are of proximal tubular origin (megalin positive), while at days 10 and 13 the majority of cysts are derived from the collecting ducts (aquaporine-2 positive). At days 21 and 28, most cysts stain positive for Tamm Horsfall protein (loop of Henle and distal convoluted tubule) or aquaporine-2 (collecting duct) (Hassane et al. unpublished).

We analyzed TGF $\beta$  (active and non-active protein) expression in the kidneys of C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice by immunohistochemistry. In wild type renal tubular epithelium, TGF $\beta$  was localized at the basolateral membrane and in the cytoplasm of all segments (Figure 1A insert) (Table 1). In cystic kidneys, the majority of the cysts was positive for TGF $\beta$ , which was expressed in the cytoplasm (Figure 1B). The expression level, however, seemed not to be increased compared to wild types.

The downstream intracellular target of TGF $\beta$ , P-Smad2, exhibits a weak expression in wild type kidneys at all ages (Figure 1C). In mutant kidneys, P-Smad2 showed strong nuclear staining in the few relatively large proximal cysts present at day 7 and in the majority of the medullary derived cysts at days 21 and 28 (Figure 1D), suggesting activation of TGF $\beta$  signaling in cystic epithelium. At days 21 and 28 many interstitial cells were found around cysts, these cells also showed nuclear localization for P-Smad2 (Figure 1D insert) and very weak cytoplasmic staining for TGF $\beta$ . To quantify these results we performed a western blot for P-Smad2 at day 21 on C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice and controls. Indeed, P-Smad2 levels were significantly increased in the mutant kidneys (6.3 times,  $p=0.0002$ ) (Figure 1Q and R).

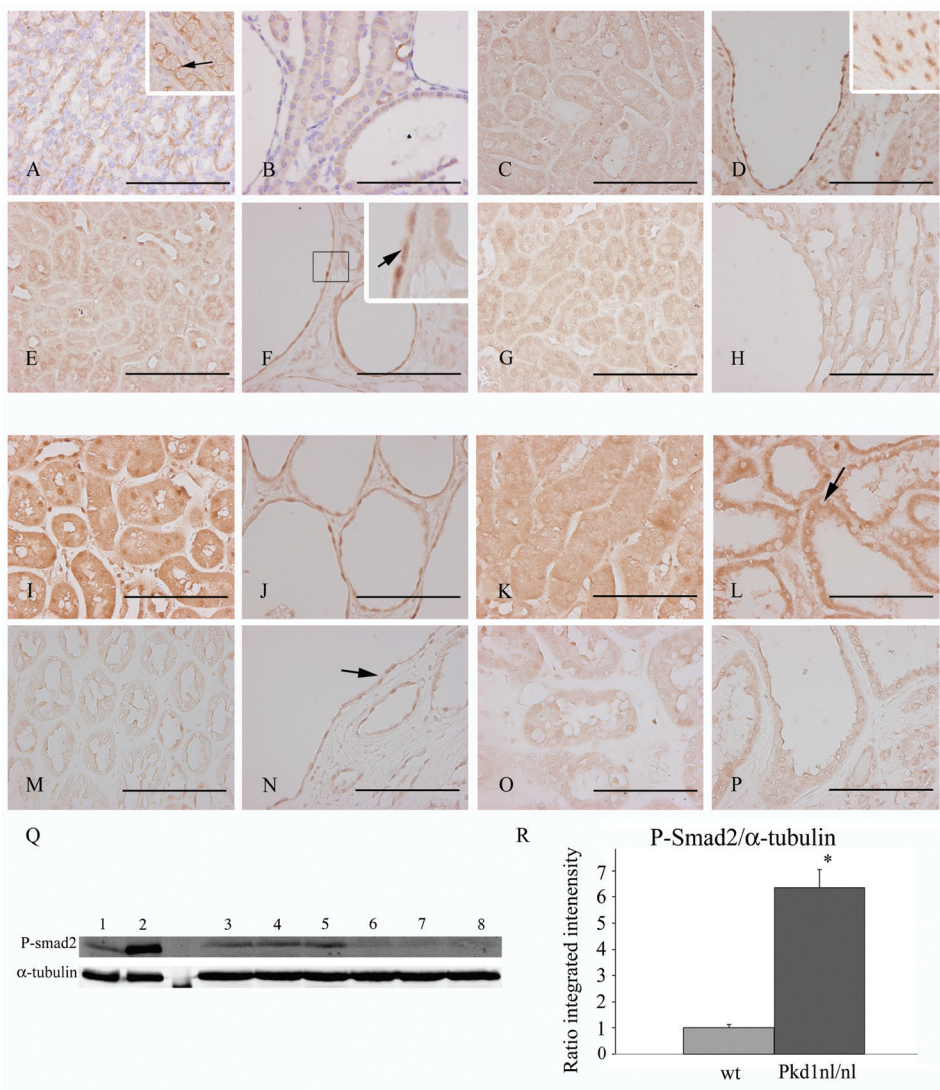


**Figure1. TGFβ signaling in *Pkd1* mutant mice and ADPKD kidneys.**

Antibody complexes in tissues are visualized using 0.04% DAB-0.5% CuSO<sub>4</sub> (brown staining). Bar = 100μm

(A) Basolateral and cytoplasmic TGFβ localization in wild type kidneys at day 21. (insert A) Basolateral TGFβ expression in 21-days-old wild type kidney. (B) Cytoplasmic TGFβ expression in cyst lining cells and weak expression in interstitial cells in C57Bl6/J-*Pkd1*<sup>nl/nl</sup> at day 21. (C) Weak cytoplasmic P-Smad2 expression in cortex in wild type kidneys at day 21. (D) Strong nuclear expression of P-Smad2 in cyst lining cells, and interstitial cells (insert) in C57Bl6/J-*Pkd1*<sup>nl/nl</sup> at day 21. (E) Weak P-Smad2 expression in medulla of 1 month old wild type kidney. (F) Strong nuclear localization of P-Smad2 (arrow) in medullary cysts and in surrounding interstitial cells of the PN4 mouse model, 30 days after *Pkd1* gene disruption. (G) Weak P-Smad1/5/8 expression in wild type kidney at the age of 1 month. (H) Weak P-Smad1/5/8 expression in cysts of PN4 mouse, 30 days after *Pkd1* gene disruption. (I) Nuclear and cytoplasmic P-Smad2 in tubules of

cortex of 5 months old wild type kidney. (J) Nuclear and cytoplasmic P-Smad2 in proximal cysts of the PN40 mouse model, 4 months after *Pkd1* deletion. (K) Nuclear and cytoplasmic P-Smad1/5/8 in cortex of 5 months old wild type kidney. (L) Decreased nuclear P-Smad1/5/8 localization (arrow) in proximal cysts of PN40 mouse model, 4 months after *Pkd1* deletion. (M) In human controls kidneys, very weak cytoplasmic and nuclear P-Smad2 in tubules. (N) In human ADPKD kidneys, strong nuclear P-Smad2 expression in cyst lining cells (arrow) and interstitial cells. (O) In human control kidneys, weak P-Smad1/5/8 expression in tubules. (P) In human ADPKD kidneys, weak P-Smad1/5/8 expression in cysts. (Q) P-Smad2 western blot with  $\alpha$ -tubulin as loading control. 1= MCF10 cells not stimulated with TGF- $\beta$  negative control. 2= MCF10 cells stimulated with TGF- $\beta$ , positive control. 3-5= C57Bl6/J-*Pkd1*<sup>nl/nl</sup> at day 21. 6-8= wild type at day 21. (R) Ratio P-Smad2/ $\alpha$ -tubulin in C57Bl6/J-*Pkd1*<sup>nl/nl</sup> and wild type kidneys. P-Smad2 is increased in C57Bl6/J-*Pkd1*<sup>nl/nl</sup>. Mean P-Smad2/ $\alpha$ -tubulin ratio of the mutant kidneys was normalized to the mean ratio in wild type kidneys which has been set to 1. \* = p<0.05

**Table 1. TGF $\beta$  and BMP signaling in C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice.**

| Age        | TGF $\beta$ , P-Smad2 and P-Smad1/5/8 expression                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
|------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Days 1-3   | <ul style="list-style-type: none"> <li>TGF<math>\beta</math> cytoplasmic in all (dilated) tubules.</li> <li>P-Smad2, weak cytoplasmic and nuclear in mature proximal (dilated) tubules, and relatively weak in tubules of the rest of the kidneys, like in wild type kidneys.</li> <li>P-Smad1/5/8, variable levels of nuclear expression in all (dilated) tubules in the kidneys, while cytoplasmic expression is weak. Same pattern found in wild type kidneys</li> </ul>                                                                                                                             |
| Day 7      | <ul style="list-style-type: none"> <li>TGF<math>\beta</math> strong basolateral and/or cytoplasmic expression in distal tubules and collecting ducts, as in wild type kidneys. Basolateral and cytoplasmic in proximal cysts.</li> <li>Increased nuclear P-Smad2 in proximal cysts and relatively weak nuclear localization in all dilated tubules in medulla.</li> <li>P-Smad2 in tubules is comparable to days 1-3, as in wild type kidneys.</li> <li>Increased nuclear P-Smad1/5/8 in proximal cysts.</li> <li>P-Smad1/5/8 in tubules is comparable to days 1-3, as in wild type kidneys.</li> </ul> |
| Days 10-13 | <ul style="list-style-type: none"> <li>TGF<math>\beta</math> basolateral and/or cytoplasmic in cysts.</li> <li>Increased nuclear P-Smad2 in few cells of some cysts.</li> <li>P-Smad2, in cortex stronger cytoplasmic expression than in medulla, as in wild type kidneys.</li> <li>P-Smad1/5/8 in tubules is comparable to days 1-7, as in wild type kidneys. In cysts, P-Smad1/5/8 is very weak in the nucleus and cytoplasm.</li> </ul>                                                                                                                                                              |
| Days 21-28 | <ul style="list-style-type: none"> <li>TGF<math>\beta</math> basolateral and/or cytoplasmic in cysts.</li> <li>Increased nuclear P-Smad2 in cystic epithelium and in interstitial cells around cysts.</li> <li>P-Smad1/5/8 in cysts at days 21 and 28 is very weak nuclear and cytoplasmic. Expression in tubules at day 21 is comparable to days 1-13, as in wild type. At day 28, nuclear and cytoplasmic expression is weak although the tubules in cortex showed a slightly stronger nuclear P-Smad1/5/8 expression compared to medulla.</li> </ul>                                                 |

*P-Smad1/5/8 Signaling at Different Stages of PKD in C57Bl6/J-Pkd1<sup>nl/nl</sup> Kidneys*  
P-Smad1/5/8 acts downstream of BMPs. Antibodies against P-Smad1/5/8 showed variable levels of expression in the nucleus in all tubular segments in wild type kidneys (Table 1). Only at day 28, the cortex showed a stronger nuclear P-Smad1/5/8 expression compared to medulla. In C57Bl6/J-*Pkd1<sup>nl/nl</sup>* mice the early proximal tubular cysts showed a relatively strong nuclear localization for P-Smad1/5/8 but the medullary derived cysts at days 21 and 28, exhibited a very weak nuclear localization (data not shown). These results indicate that there is no strong activation of P-Smad1/5/8 signaling during cyst formation and cyst growth.

*P-Smad2 and P-Smad1/5/8 Expression at different stages in kidneys of young and adult inducible Pkd1-deletion mice*

To further analyze TGFβ signaling during cyst formation we analyzed renal tissues from a conditional and inducible *Pkd1*-deletion mouse model.<sup>16</sup> In this model *Pkd1* gene deletion is induced by tamoxifen administration. Timing of *Pkd1* disruption affects the phenotype. Tamoxifen administration at day 4 (PN4) induces 5% *Pkd1* gene disruption, as measured by extension MLPA (eMLPA)<sup>17</sup>. This results in a limited number of fast progressing cysts within one month. These cysts originate mainly from distal tubules and to less extent from collecting ducts. These cysts as well as the interstitial cells showed increased nuclear localization of P-Smad2 (Figure 1E and F), while levels and expression pattern of P-Smad1/5/8 were comparable to wild type kidneys (Figure 1G and H) (Table 2).

**Table2. TGFβ and BMP signaling in *Pkd1*-deletion mice.**

| Mouse             | P-Smad1/5/8 and P-Smad2 expression                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
|-------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| PN4+<br>1 month   | <ul style="list-style-type: none"> <li>Increased nuclear P-Smad2 in distal cysts and interstitial cells.</li> <li>P-Smad1/5/8, weak nuclear as well as cytoplasmic expression in tubules. Same pattern as in wild type kidneys.</li> <li>P-Smad1/5/8 very weak cytoplasmic in distal cysts.</li> </ul>                                                                                                                                                                                                                                                                                          |
| PN40+<br>4 months | <ul style="list-style-type: none"> <li>P-Smad2 nuclear and cytoplasmic expression in cysts in cortex. Same pattern as in cortex of wild type kidneys.</li> <li>P-Smad2 variable levels of nuclear expression in outer medulla. Strong nuclear expression in inner medulla. Same pattern as in wild type kidneys</li> <li>Decreased nuclear P-Smad1/5/8 in most proximal dilated tubules and cysts. A few proximal cysts showed rather strong nuclear expression.</li> <li>P-Smad1/5/8 very weak cytoplasmic and nuclear expression in medulla. Same pattern as in wild type kidneys.</li> </ul> |

Deleting *Pkd1* at day 40 (PN40) results in a cystic phenotype showing many dilated tubules in the cortex and medulla after 3 months and proximal cysts after 4 months. Nuclear localization of P-Smad2 was detected in cysts and dilatations of the outer cortex and less in the inner cortex at 3 and 4 months after gene disruption. The expression level and pattern were comparable to wild type kidneys (Figure 1I and J) (Table2). On the other hand, the expression of P-Smad1/5/8, was almost absent in the nucleus of most proximal

cysts and dilatations, while in wild type kidneys weak nuclear and cytoplasmic signal was observed, suggesting increased Smad2-mediated gene expression in cystic epithelium (Figure 1K and L). At the early stages, when no dilatations or cysts were observed, P-Smad2 and P-Smad1/5/8 expression levels and patterns were comparable to wild types (not shown).

#### *P-Smad2 and P-Smad1/5/8 in ADPKD Patients*

The kidneys of 4 healthy individuals and 9 end stage ADPKD-patients were analyzed as well. In general, expression of P-Smad2 and of P-Smad1/5/8 was weak in control kidneys (Figure 1M and O), except for some tubules that showed strong nuclear and cytoplasmic signals for both groups of Smads. In patients, strong nuclear staining for P-Smad2 was frequently seen in groups of cysts. In these areas cysts as well as the surrounding fibrotic tissue stained positive for P-Smad2 (Figure 1N). For P-Smad1/5/8 the majority of the cysts showed only weak nuclear and cytoplasmic staining (Figure 1P). Only a few cysts were positive for P-Smad1/5/8. These results are in line with expression pattern we found in our mouse models and support the notion that Smad2-signaling is increased in more advanced cystic kidneys, while Smad1/5/8-regulated signaling is hardly activated.

#### *Expression of Target Genes During Early and Progressive Stages of PKD.*

To determine the expression of TGF $\beta$  target genes, transcript levels of matrix metalloproteinase-2 (*MMP-2*), Plasminogen activator inhibitor 1 (*PAI-1*), collagen type 1 and EDA-fibronectin were measured in 21-days-old C57Bl6/J-*Pkd1*<sup>nl/nl</sup> kidneys.<sup>25-29</sup> Also expression of *MMP-14*, an activator of *MMP-2* was analyzed.<sup>30</sup> Expression of all these genes was significantly increased in C57Bl6/J-*Pkd1*<sup>nl/nl</sup> kidneys (Figure 2A). To confirm elevated *MMP-2* expression at the protein level, we did immunohistochemical analyses on C57Bl6/J-*Pkd1*<sup>nl/nl</sup> kidneys at different ages. In wild type kidneys *MMP-2* expression was almost absent (Figure 3A). In mutant kidneys, however, *MMP-2* expression increased in cystic epithelium as cyst formation progresses (Figure 3B, arrow), starting at day 7 in proximal cysts. These cysts also showed increased nuclear P-Smad2 expression.

Furthermore, we measured expression of TGF $\beta$  target genes in *Pkd1* deletion mouse models (PN4 and PN40) at initiation and more advanced stages of PKD. In both models expression was significantly increased only at more advanced stages of the disease but not at the earliest time points (Figure 2B and C). In addition, the levels of one BMP target gene, Inhibitor of DNA binding/differential-1 (*Id-1*),<sup>31</sup> was measured as well. For this gene, transcript levels were comparable to wild type mice at early and more advanced stages of the different models (Figure 2A and B). These data are in line with the immunohistochemical data that indicate activation of TGF $\beta$  signaling, but not BMP signaling, at late stage PKD.

#### *Fibrosis in Pkd1 Mutant Mouse Models*

Renal fibrosis is accompanied by an increase in interstitial fibroblasts and by extracellular matrix deposition, e.g. collagen.<sup>32</sup> Furthermore, TGF $\beta$  can transform fibroblasts to myofibroblasts, which can be identified as interstitial cells expressing  $\alpha$ -smooth muscle actin.<sup>33</sup> Therefore we analyzed the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), collagen (sirius red) and the fibroblast marker Fibroblast Specific Protein-1 (FSP-1).<sup>34</sup> At days 1 through 7, no fibrosis was detected in C57Bl6/J-*Pkd1*<sup>nl/nl</sup> kidneys.

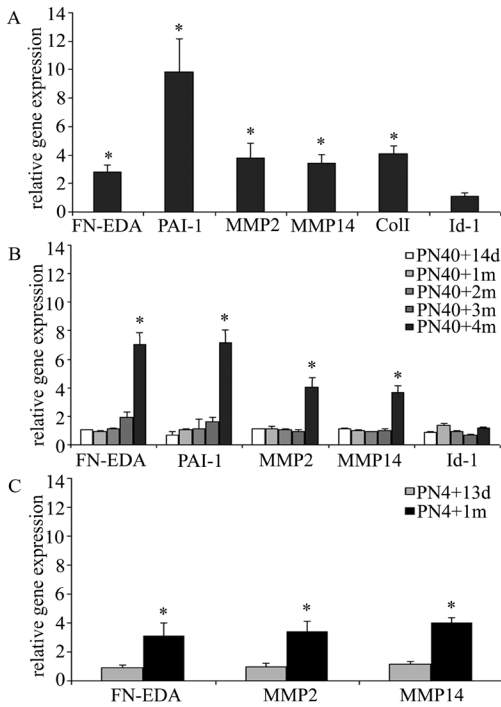


**Figure 2. Expression levels of BMP and TGFβ target genes.**

(A) Transcript levels of *Id-1*, a BMP target gene, and 4 TGFβ targets genes, EDA-fibronectin (*FN-EDA*), *PAI-1*, *MMP-2*, and collagen type I (*Coll*), as well as levels of *MMP-14*, an activator of *MMP-2*, were determined by RT-MLPA in 21-days-old C57Bl6/J-*Pkd1<sup>nl/nl</sup>* and wild type kidneys. For each gene, transcript levels of the mutant kidneys were normalized to the mean ratio of the same gene in wild type kidneys, which has been set to 1. In mutant kidneys expression of the TGF-β target genes is significantly elevated while expression of BMP target *Id-1* is comparable to wild type kidneys. (B) TGFβ and BMP target genes in *Pkd1* deletion mouse PN40. The expression of TGFβ target genes starts to increase 3 months after deletion of the *Pkd1* gene and are significantly increased after 4 months. *Id-1* expression is not significantly altered at any time

point. (C) *Pkd1*-deletion mouse PN4. The expression of TGFβ target genes is not increased 14 days after *Pkd1* disruption, however, after 30 days TGFβ target genes are increased. \* =  $p < 0.05$

At days 10 and 13, α-SMA positive myofibroblasts were found around most cysts at the cortico-medullary region, while collagen started to accumulate around a few of the cysts. The amount of fibrotic tissue and the number of cysts surrounded by α-SMA positive cells and collagen increased at days 21 and 28 (Figure 3C-F). The tissue area occupied by α-SMA positive cells was quantified at day 28, revealing a significant increase of α-SMA in cortex and medulla (cortex  $2.39 \pm 0.77$  vs.  $16.73 \pm 3.47$   $p=0.005$ ; medulla  $2.07 \pm 1.47$  vs.  $23.05 \pm 4.28$   $p=0.04$ ) (Figure 4A and B). Collagen deposition was also significantly increased (cortex  $7.48 \pm 5.09$  vs.  $29.57 \pm 0.19$   $p=0.004$ ) (Figure 4E and F).

The inducible *Pkd1*-deletion mouse model, PN4+1m, showed a similar result for α-SMA as C57Bl6/J-*Pkd1<sup>nl/nl</sup>*, in cortex and medulla (cortex  $2.39 \pm 0.77$  vs.  $9.24 \pm 1.89$ ,  $p=0.01$ ; medulla  $2.07 \pm 1.47$  vs.  $18.76 \pm 3.35$   $p=0.004$ ) (Figure 3G and 4A and B). Also collagen deposition was significantly increased in medulla (medulla  $17.37 \pm 8.38$  vs.  $41.91 \pm 1.80$   $p=0.02$ ) (Figure 4E and F). The inducible *Pkd1*-deletion mice PN40+4m, showed a thin layer of α-SMA positive myofibroblasts around some cysts in cortex (Figure 3H), while the outer medulla with many dilated tubules showed an increased number of α-SMA positive cells (Figure 3I and J).

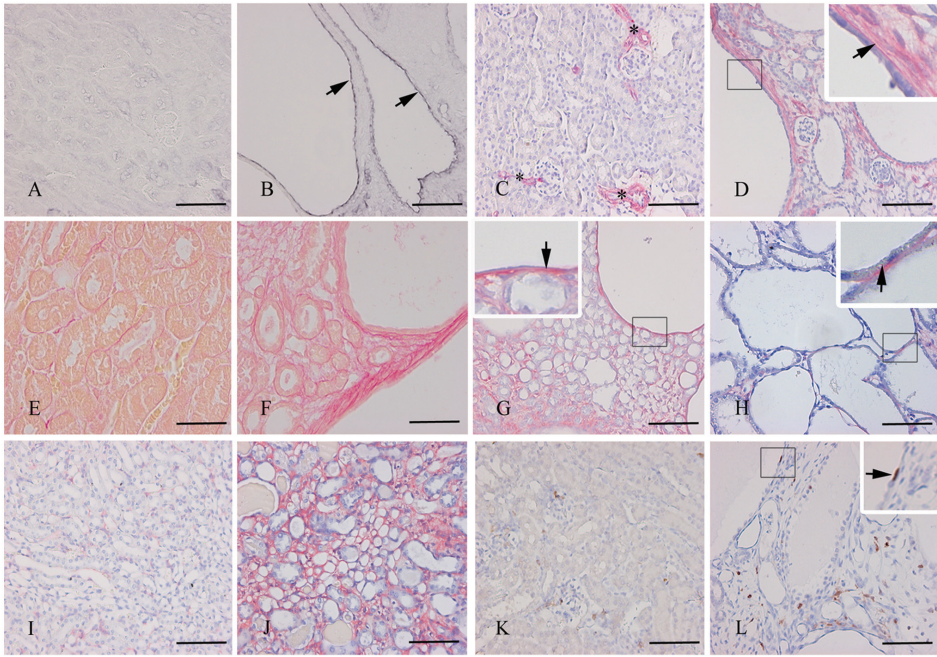


**Figure 3. Fibrosis in *Pkd1* mutant kidneys.**

(A-B) staining MMP-2 (black staining) (C-D; G-J) staining for  $\alpha$ -SMA (pink staining) (E-F) staining for sirius red (red staining) (K-L) staining for FSP-1 (brown staining). C&D and G-L are counterstained with hematoxylin. (A) Virtually no MMP-2 expression is present in wild type kidneys at day 21. (B) Increased MMP-2 in cyst-lining cells (arrows) in C57Bl6/J-*Pkd1*<sup>nl/nl</sup> at day 21. (C) In cortex of 1 month old wild type kidneys only blood vessels show  $\alpha$ -SMA positive cells (\*=blood vessel). (D)  $\alpha$ -SMA positive cells particularly around cysts (arrow in insert) and dilated tubules in cortex of 1 month old C57Bl6/J-*Pkd1*<sup>nl/nl</sup> kidney. (E) Collagen deposition, visualized by Sirius red staining, in wild type kidney at day 21. (F) Collagen deposition is increased, particularly around cysts, in cystic kidneys of C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice, shown at day 21. (G)  $\alpha$ -SMA positive cells in cortex of PN4 *Pkd1*-deletion mouse kidney at one month after gene disruption. Cyst lining cells are positive (arrow in insert) as well as interstitial cells surrounding cysts and tubules. (H)  $\alpha$ -SMA positive interstitial cells in the cortex of PN40 *Pkd1*-deletion mouse kidney (arrow in insert), 4 months after gene disruption. (I) In medulla of 1 month old wild type kidneys only blood capillaries show  $\alpha$ -SMA positive cells. (J) Many  $\alpha$ -SMA positive interstitial cells in the medulla of PN40 *Pkd1*-deletion mouse kidney, 4 months after gene disruption. (K) A few FSP-1 positive cells in 1 month old wild type kidney. (L) Low number of FSP-1 positive cells in 1 month old C57Bl6/J-*Pkd1*<sup>nl/nl</sup> kidney. A few flat cystic epithelial cells showed FSP-1 (arrow). Bar = 100 $\mu$ m

Both, in cortex and medullar  $\alpha$ -SMA expression was significantly increased in mutants compared to wild types (cortex  $1.64 \pm 0.22$  vs.  $7.73 \pm 1.73$   $p=0.04$ ; medulla  $3.36 \pm 2.20$  vs.  $31.14 \pm 0.68$   $p=0.03$ ) (Figure 4A and B). Collagen deposition was significantly increased in medulla of PN40+4m mice (medulla  $1.84 \pm 0.24$  vs.  $4.19 \pm 0.59$   $p=0.035$ ) (Figure 4E and F).

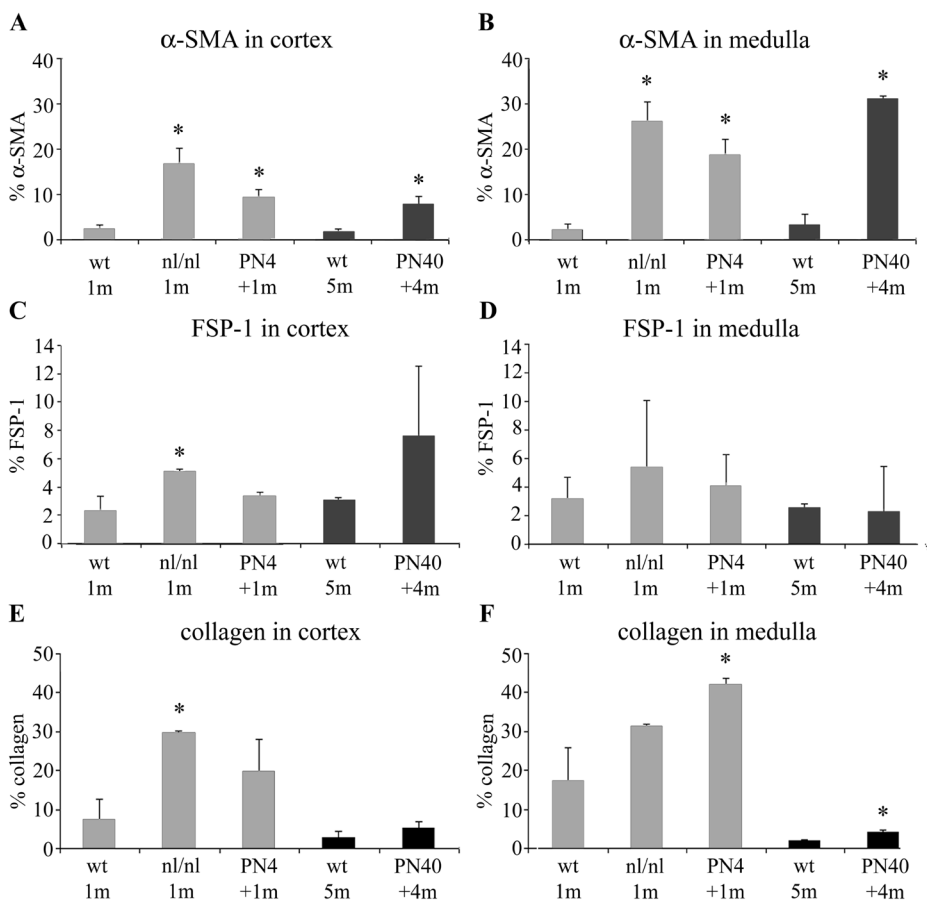


**Figure 4. α-SMA, FSP-1 and collagen expression in *Pkd1* mutant kidneys.**

Grey bars = 1 month old, black bars = 5 months old.

(A-B) α-SMA in cortex and medulla of C57Bl6/J-*Pkd1*<sup>nl/nl</sup> and PN4+1m compared to wild type 1 month (wt 1m), and PN40+4m compared to wild type 5 months (wt 5m). Significant increase of α-SMA in cortex and medulla in all mice. (C57Bl6/J-*Pkd1*<sup>nl/nl</sup>: cortex  $2.4 \pm 0.77$  vs.  $16.7 \pm 3.5$ ,  $p=0.005$ ; medulla  $2.1 \pm 1.5$  vs.  $23.1 \pm 4.3$ ,  $p=0.04$ ) (PN4+1m: cortex  $2.4 \pm 0.77$  vs.  $9.2 \pm 1.9$ ,  $p=0.01$ ; medulla  $2.1 \pm 1.5$  vs.  $18.8 \pm 3.3$ ,  $p=0.004$ ) (PN40+4m: cortex  $1.6 \pm 0.2$  vs.  $7.7 \pm 1.7$ ,  $p=0.04$ ; medulla  $3.4 \pm 2.2$  vs.  $31.1 \pm 0.7$ ,  $p=0.03$ ). \* =  $p < 0.05$

(C-D) FSP-1 in cortex and medulla of C57Bl6/J-*Pkd1*<sup>nl/nl</sup> and PN4+1m compared to wild type 1 month (wt 1m), and PN40+4m compared to wild type 5 months (wt 5m).

FSP-1 was significantly increased in cortex of C57Bl6/J-*Pkd1*<sup>nl/nl</sup> (C57Bl6/J-*Pkd1*<sup>nl/nl</sup>: cortex  $2.33 \pm 1.03$  vs.  $5.05 \pm 0.07$ ,  $p=0.044$ ). \* =  $p < 0.05$

(E-F) collagen in cortex and medulla of C57Bl6/J-*Pkd1*<sup>nl/nl</sup> and PN4+1m compared to wild type 1 month (wt 1m), and PN40+4m compared to wild type 5 months (wt 5m). Collagen deposition was significantly increased in all three models (C57Bl6/J-*Pkd1*<sup>nl/nl</sup>: cortex  $7.48 \pm 5.09$  vs.  $29.57 \pm 0.19$ ,  $p=0.004$ ) (PN4+1m: medulla  $17.37 \pm 8.38$  vs.  $41.91 \pm 1.80$ ,  $p=0.02$ ) (PN40+4m: medulla  $1.84 \pm 0.24$  vs.  $4.19 \pm 0.59$ ,  $p=0.035$ ) \* =  $p < 0.05$

Expression of FSP-1 was significantly increased only in cortex of C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice (cortex  $2.33 \pm 1.03$  vs.  $5.05 \pm 0.07$   $p=0.044$ ). (Figure 3K and L and 4C and D). In general we see in all *Pkd1* mutants, that most  $\alpha$ -SMA positive fibroblasts were found as a second layer around the cystic epithelial cells (Figure 3D insert). The majority of cysts is surrounded by  $\alpha$ -SMA positive cells, while only now and then a FSP-1 positive cell could be seen in the interstitium around cysts. In addition, a few flat cystic epithelial cells showed FSP-1 or  $\alpha$ -SMA expression, suggesting that if epithelial-to-mesenchymal-transition (EMT) contributes to PKD and fibrosis, it is only to a very low extent (Figure 3G and L). These flat FSP-1 or  $\alpha$ -SMA positive cyst lining epithelial cells were more pronounced in the PN4 and less in the PN40 inducible mice and C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice.

## Discussion

Cystogenesis is characterized by epithelial cell proliferation, excessive secretion of solute and fluid into the cysts lumen and accumulation of matrix proteins in the renal interstitium, resulting into loss of functional renal tissue and interstitial fibrosis. TGF $\beta$  is the major fibrogenic cytokine that has been studied in detail in renal interstitial diseases.<sup>35</sup> In this study, we show that TGF $\beta$  signaling is not increased in the initial stage of cyst formation, but is increased during cyst expansion in more advanced stages of PKD in *Pkd1* mutant mouse models and in ADPKD patient kidneys, which are characterized by progressive cysts formation and fibrosis. Increased nuclear localization of P-Smad2 confirms activation of the TGF $\beta$  signaling pathway and is consistent with the elevated mRNA levels of TGF $\beta$  target genes, like EDA-fibronectin, collagen type I, PAI-1 and MMP-2, at later stages in the mouse models. These data are in line with studies on expression profiling of end-stage ADPKD kidneys and kidneys of *Han:SPRD-Cy/+* rats, revealing upregulation of genes involved in extracellular matrix synthesis, EMT and fibrosis.<sup>13,36</sup> Furthermore, expression of TGF $\beta$  and TGF $\beta$  regulated genes is low in young *Han:SPRD-cy/+* rats but increases with progression of the disorder.<sup>37</sup> In our *Pkd1* mouse models, cytoplasmic TGF $\beta$  expression as well as nuclear localization of P-Smad2 were detected in cystic epithelial cells as well as in interstitial cells suggesting that both cell types synthesize and respond to TGF $\beta$ . Indeed, both interstitial cells as well as epithelial cells are sources of increased TGF $\beta$  production and are major sites of matrix protein overproduction.<sup>38-42</sup>

During epithelial-to-mesenchymal transition (EMT) epithelial characteristics are lost and a mesenchymal phenotype is acquired.<sup>43</sup> The *Pkd1* mutant mice sometimes showed flat cyst lining cells that were positive for FSP-1 or  $\alpha$ -SMA. This phenomenon was more obvious in PN4 induced mice than in the other models. Differences in the stage of progressiveness of PKD, the developmental stage of the kidneys at the moment of gene-disruption and/or the nephron-segment from which cysts are derived (distal tubules in PN4) may play a role in this process. Overall, the proportion of FSP-1 or  $\alpha$ -SMA positive epithelial cells is small. Furthermore, only incidentally single cells seemed to disconnect from the neighboring cells towards lumen or matrix. These data suggest that if EMT contributes to PKD and fibrosis, this is only to a very low extent.

In the *Pkd1* mutant mouse models presented here we observed relatively mild fibrosis with minor accumulation of collagen compared to human cystic kidneys. In these models, cyst formation progresses relatively fast and kidney failure occurs before the

development of progressive fibrosis. Similar to these mice, mild fibrosis was found in *wpk* and homozygous *Han:SPRD-cy/cy* rats and *jcpk* mice, which also show a rapidly progressive cyst formation.<sup>44-46</sup> On the other hand, slowly progressive forms of PKD, such as heterozygous *Han:SPRD-cy/+* rats, and *pcy* models and human ADPKD, develop severe renal fibrosis.<sup>46-47</sup>

Interstitial fibrosis is not only caused by increased matrix synthesis, but inadequate matrix degradation also contributes to the fibrogenic process.<sup>32</sup> TGF $\beta$  is involved in the regulation of both processes as it activates expression of matrix molecules (e.g. collagen I and EDA-fibronectin) and inhibitors of matrix degrading enzymes, thereby resulting in matrix accumulation. PAI-1, a negative regulator of MMP's, is elevated in several acute and chronic renal disease.<sup>48-49</sup> Upregulation of PAI-1 promoter activity by TGF $\beta$  signaling is believed to be a major mechanism through which TGF $\beta$  downregulates ECM degradation.<sup>50</sup> Indeed, PAI-1 transcript levels were approximately 8-10 times increased in advanced stages in the *Pkd1* mutant mice. Levels of another Smad2 target involved in matrix remodeling, MMP-2 and its activator MMP-14, are 3 to 4 times increased in *Pkd1* mutant mice, suggesting a complex regulation of matrix remodeling with an imbalance towards accumulation of matrix proteins.

The importance of BMP-7 for renal fibrosis was shown by several studies demonstrating that administration of BMP-7 reduces fibrotic tissues in various animal models of renal failure, by antagonizing TGF $\beta$ .<sup>51-54</sup> In C57Bl6/J-*Pkd1*<sup>nl/nl</sup> and young induced *Pkd1* deletion mice (PN4), P-Smad1/5/8 is hardly expressed in cysts and its expression is a reflection of the expression pattern found in wild type kidneys. This was accompanied by increased P-Smad2 activation in the same cysts in these mouse models. However at older ages in adult induced *Pkd1*-deletion mice (PN40), in the majority of cysts P-Smad1/5/8 is excluded from nuclei of proximal cysts compared to the wild type kidneys, while P-Smad2 remained localized in the nuclear compartment. This imbalance towards increased TGF $\beta$  signaling observed in all three mouse models provides a rationale to test TGF $\beta$  antagonists in PKD, at mild to advanced stages of PKD.

In conclusion, TGF $\beta$  signaling is strongly increased at more advanced stages of polycystic kidney disease but is hardly altered at the early stage of the disease, suggesting that the TGF $\beta$  signaling pathway is probably not implicated in initial steps of cyst formation. Elevated TGF $\beta$  signaling overlaps with and stimulates expression of matrix remodeling proteins, which are characteristic for fibrosis. These results suggest that targeting TGF $\beta$  signaling may reduce or inhibit fibrosis, thereby retarding disease progression.

## Methods

### Mice

The C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice were generated as previously described in detail.<sup>15</sup> Offspring were backcrossed with C57Bl6/J mice for 12 generations, resulting in an almost 100% C57Bl6/J background. We analyzed C57Bl6/J-*Pkd1*<sup>nl/nl</sup> at different ages (days 1, 3, 7, 10, 13, 21, and 28). The inducible *Pkd1*-deletion mice (tam-KspCad-CreERT2;*Pkd1*<sup>del2-11/lox2-11</sup>) and tamoxifen treatment have been described previously.<sup>16</sup> Adult mice, were treated with 5 mg tamoxifen, at post-natal day 40 (PN40), for three consecutive days, and sacrificed 14 days and 1, 2, 3, and 4 months later (PN40+14d, PN40+1m, etc). Young inducible mice received tamoxifen via breast-feeding from the mother starting at post-natal day 4 (PN4). These mice were sacrificed 13 days (PN4+13d) and one month (PN4+1m) later. Somatic *Pkd1* gene disruption was confirmed by extension

Multiplex Ligation-dependent Probe Amplification (eMLPA) described previously.<sup>17</sup>

All experiments with mice were approved by the Animal Care and Use Committee of Leiden University and by the Commission for Biotechnology in Animals of the Dutch Ministry of Agriculture.

#### *Human Tissue Samples of Normal and Cystic Kidneys*

Renal tissues from ADPKD patients with chronic renal failure and control renal tissues were fixed in 4% formalin and embedded in paraffin. Control tissues were isolated from donor kidneys intended for transplantation, but not suitable due to technical reasons. In total 4 control samples and 9 ADPKD cystic tissue samples of patients (8 with known mutations in or positive linkage to *PKD1*) were analyzed.

All human tissue samples were analyzed following procedures approved by the ethical committee (institutional review board) of the Leiden University Medical Center.

#### *Antibodies*

The following primary antibodies were used: P-Smad1/5/8 and P-Smad2 (1:500; rabbit polyclonal antibodies),<sup>18-20</sup> Fibroblast Specific Protein-1 (FSP-1, A5114; 1:800; rabbit polyclonal antibody; Dako, Heverlee, Belgium), matrix metalloproteinase-2 (MMP-2; mouse anti-human MMP-2 monoclonal antibody (Ab4), 1:4, clone 75-7F7; Calbiochem, Amsterdam, the Netherlands),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA/AF (1A4) A5691; 1:100; mouse monoclonal antibody; Sigma, Zwijndrecht, the Netherlands), total TGF $\beta$  1 and 2 (active and inactive) sc-146; 1:1600; rabbit polyclonal antibody, Santa Cruz, Heidelberg, Germany. Of note: variations in between batches were observed). Secondary antibodies: mouse envision/HRP (not diluted; Dako, Heverlee, Belgium), rabbit-anti-goat Ig/HRP (1:100; Dako, Heverlee, Belgium).

#### *Immunohistochemistry*

Immunohistological analysis was performed on 24 C57Bl6/J-*Pkd1*<sup>nl/nl</sup> and 22 wild type kidneys of different ages. In addition, we analyzed kidneys of inducible *Pkd1*-deletion mice with *Pkd1* gene disruption at post-natal day 4 (PN4) or post-natal day 40 (PN40). We analyzed two *Pkd1*-deletion mice (PN4) and four age-matched control kidneys, at the age of 1 month, as well as twelve *Pkd1*-deletion mice (PN40) and 12 age-matched wild type kidneys, at different time points after *Pkd1* gene disruption. The kidneys were fixed in 4% formalin and embedded in paraffin. After de-paraffinization, sections were stained using standard procedures.<sup>21</sup> All antibodies were incubated over night at 4°C and the antibody complexes were visualized using 0.04% diaminobenzidine-tetra-hydrochloride (DAB) with 0.5% CuSO<sub>4</sub> (brown precipitate) or 0.7% NiCl<sub>2</sub> (black precipitate), except for  $\alpha$ -SMA/AF (conjugated with alkaline phosphatase) that was incubated for one hour at room temperature and visualized by fast red.

Antigen-retrieval was done for P-Smad2 and MMP-2 using citric acid buffer (0.01 M, pH 6.0) at 100°C for 10 min, and for P-Smad1/5/8 and FSP-1 sections were incubated in Tris:EDTA (10:1 mM) at 100°C for 10 min. Specificity of immunoreactive signals for TGF $\beta$  was established by competition with recombinant human TGF $\beta$ 1 (Peprotech, London, UK); 10x and 100x surplus). For histological analyses kidney sections were stained with hematoxylin-eosin (HE) and Sirius red (stains total collagen).<sup>22</sup>

An extensive detailed analysis of all stainings has been done by two individuals. In addition two other individuals also judged the stainings. For FSP-1,  $\alpha$ -SMA and sirius red we randomly photographed six areas of the cortex and medulla at the magnification of 200x. FSP-1 was quantified by counting the percentage of FSP-1-positive cells. Between 500 and 900 epithelial and interstitial cells were counted per section; glomeruli were excluded. The areas stained for  $\alpha$ -SMA and sirius red were quantified using software ImageJ (available at <http://rsb.info.nih.gov/ij/>). The proportion of collagen, FSP-1 and  $\alpha$ -SMA of the wild type and mutant mice were compared with an independent sample 2-tailed T-test. A P-value of <0.05 (2 tailed) was considered significant.

#### *Western blot*

To quantify P-Smad2 expression we performed a western blotting on kidneys of 3 wild type and 3 C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice at the age of 21 days. Tissues were homogenized using MagNALyser (Roche Diagnostics; 2 times 20 seconds at 7000rpm) in RIPA buffer (50mM Tris HCL PH7.4, 150mM NaCl, 1mM EDTA) with 2mM NaF and 1mM Na<sub>2</sub>VO<sub>4</sub> (Phosphatase inhibitors) and protease inhibitor cocktail (Roche, Woerden, The Netherlands). This was followed by 3 times 5 seconds sonification on ice. RIPA buffer with 2 times detergent was added to the lysates to get final concentrations of 1x RIPA with 1%DOC, 1%NP40, 1%TritonX100, and incubated for 45 min at 4°C. Finally, lysates were centrifuged 10 min at 14.000g and supernatants were used for the western; 100  $\mu$ g protein was loaded for each sample. Western blotting was performed as described previously.<sup>4</sup> Blots were blocked by 0.25x SEA blocking buffer (Pierce, Rockford, USA) in PBS. Primary antibodies include anti- $\alpha$ -tubulin (DM1A; CP06; 1:2000; mouse monoclonal antibody; Calbiochem, Amsterdam, The Netherlands) and P-Smad2/3 (1:10.000; rabbit polyclonal antibodies).<sup>18</sup> Secondary antibodies include anti-IgG-rabbit (Fc) IRDye

800CW (1:7000; Tebu bio, Heerhugowaard, the Netherlands) and anti-IgG-mouse Dylight 680 (1:7000, Tebu bio, Heerhugowaard, the Netherlands). Blots were visualized and scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences). The values of wild type and mutant mice were compared with an independent sample 2-tailed T-test. A P-value of <0.05 (2-tailed) was considered significant.

### *mRNA Quantification*

Transcript levels of matrix metalloproteinase-2 and -14 (MMP-2 and -14), Plasminogen activator inhibitor-1 (PAI-1), collagen type 1 and EDA-fibronectin, were determined by Reverse Transcriptase Multiplex Ligation dependent Probe Amplification (RT-MLPA). We analyzed 4 C57Bl6/J-*Pkd1*<sup>nl/nl</sup> and 3 wild type kidneys (21 days old). In addition, 12 young induced *Pkd1* deletion (PN4) and 12 control kidneys at two time points (13 and 30 days after *Pkd1* deletion) and 28 adult induced *Pkd1* deletion (PN40) and 25 control kidneys at different time points (14 days and 1, 2, 3, and 4 months after *Pkd1* deletion) were analyzed. The RT-MLPA reactions were performed as previously described.<sup>23,24</sup> Briefly, kidneys were homogenized and total RNA was isolated. From an aliquot of 200-300 ng total RNA, cDNA was synthesized. Two separate hybridization reactions were performed with this cDNA. To each sample, MLPA probe mix and SALSA MLPA buffer were added (MRC-Holland, Amsterdam, the Netherlands), and the samples were incubated at 95°C for 1 min followed by 60°C for 4 hours. Sequences of oligonucleotide probes, primers and competitors are available at request. The ligation of the annealed probes was performed at 54°C for 10-15 min. The ligase enzyme was inactivated by heating at 98°C for 5 min, and the ligation products were amplified by PCR using SalsaTaq (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands). The amplified samples were mixed with Hi-Di Formamide containing GeneScan-500 ROX size standard (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands) heated for 5 min at 95°C and run on a 3730 DNA analyzer (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands). Data were analyzed using GeneScan Analysis 3.5 and Microsoft Excel software. For situations in which off-scale signals needed to be reduced, competitor oligos were applied. The expression of the house keeping genes *Ywhaz* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide) and *Hprt* (hypoxanthine guanine phosphoribosyl transferase) served as reference for gene expression. Peak ratios of the target genes and house keeping genes were calculated, and results of the mutant and wild-type kidneys were normalized to the median of wild type kidneys of the same age, which was set to 1. The MLPA values of the wild type and mutant mice were compared with an independent sample 2-tailed t-test. A p-value of <0.05 (2-tailed) was considered significant.

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## Chapter 4

### **Pathogenic sequence for dissecting aneurysm formation in a hypomorphic polycystic kidney disease 1 mouse model.**

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Arterioscler Thromb Vasc Biol. 2007 Oct;27(10):2177-83

## Abstract

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a multi-system disorder characterized by progressive cyst formation in the kidneys. Serious complications of ADPKD are intracranial and aortic aneurysms. The condition is mainly caused by mutations in the *PKD1* or *PKD2* gene. We have carefully analyzed vascular remodeling in hypomorphic *Pkd1<sup>nl/nl</sup>* mouse model with dissecting aneurysms in the aorta.

Quantitative real-time polymerase chain reaction revealed that in the aorta the expression of normal *Pkd1* is reduced to approximately 26%. Using (immuno)histochemistry we have characterized the pathogenetic sequence for dissecting aneurysm formation. The aorta shows regions with accumulation of matrix components between the elastin lamellae. This is followed by increased numbers of smooth muscle cells and locally weakening of the media. In the intima, accumulation of matrix components and detachment of endothelial cells from the elastin lamellae results in a tear. The combination of weak media and a tear in the intima leads to rupture of the vessel wall resulting in intramural bleeding. The *Pkd1<sup>nl/nl</sup>* mouse reveals that polycystin1 is implicated in maintenance of the vessel wall structural integrity, and it is a useful model for dissecting aneurysm formation studies.

## Introduction

Autosomal Dominant Polycystic Disease (ADPKD) is one of the most common genetic diseases. ADPKD affects 1 in 1000 individuals and is characterized by progressive development of renal cysts and an increased risk of aneurysm formation. The prevalence of cerebral aneurysms in ADPKD patients is approximately 10-fold higher than in the general population and about 27% among ADPKD patients with a family history for aneurysms.<sup>1,2</sup> The few clinical studies done to determine the frequency of aortic aneurysms in ADPKD, suggest a prevalence varying from 1% to 10% in ADPKD patients.<sup>3-5</sup> Furthermore, a gene expression profile study revealed a decrease of *PKD1* expression in dissected human aortas.<sup>6</sup> ADPKD has been associated with mutations in the *PKD1* or *PKD2* gene, encoding the proteins polycystin-1 and polycystin-2. Both genes are expressed in the smooth muscle cells (SMCs) and endothelial cells (ECs) of the blood vessel.<sup>7-9</sup> At present, however, hardly any histopathologic analyses on dissections and aneurysms in ADPKD have been performed.

Several *Pkd1* and *Pkd2* knock-out mouse models have been generated, however knock-out embryos die at embryonic days 13.5 to 14.5 from a primary cardiovascular defect.<sup>9-12</sup> These embryos show edema, focal vascular leaks, and hemorrhage. The fact that these mice die in utero withholds the possibility to study aneurysm formation in ADPKD.

Recently, we have generated a *Pkd1* hypomorphic mouse model, *Pkd1<sup>nl/nl</sup>*, with reduced *Pkd1* transcripts in the kidneys.<sup>13</sup> *Pkd1<sup>nl/nl</sup>* mice are viable, and the majority of the animals die within 1 to 2 months after birth. These mice show dissecting aneurysms and cysts in kidneys, liver, and pancreas, which is in line with the pathogenic features found in the human ADPKD phenotype.

*Pkd1<sup>nl/nl</sup>* mice are the first viable ADPKD mice with spontaneous dissecting aneurysm formation in the aorta enabling studies on vascular remodeling in ADPKD. In this study we have carefully characterized dissecting aneurysm formation in these hypomorphic mice. Reduced levels of *Pkd1* transcripts measured in the aorta indicate a direct association between *Pkd1* and vessel wall integrity.

Results

Analysis of Aortic *Pkd1* mRNA Levels

We used *Pkd1*<sup>nl/nl</sup> mice containing a *loxP*-flanked neomycin selection-cassette in intron 1 of the *Pkd1* gene. We previously have shown that a fragment of the neomycin cassette is incorporated in a fraction of the *Pkd1* transcripts by alternative splicing in the kidneys, resulting in a frame shift and predicted to result in an early premature translation stop.<sup>13</sup> This results in a reduction of the normal *Pkd1* expression. Here we determined the level of normal *Pkd1* in the aortas of hypomorphic and wild type mice with a real-time PCR assay. In mutant mice, the expression of normal *Pkd1* is reduced to approximately 26% in the aortas and 37% in the kidneys of the same mice (Figure 1A). The total *Pkd1* expression (normal plus mutant *Pkd1*) in the *Pkd1*<sup>nl/nl</sup> mice is about 1.5 and 2.7 times-fold increased in aorta and kidney, respectively (Figure 1B). In aortas of *Pkd1*<sup>nl/nl</sup> mice the ratio of normal versus total *Pkd1* expression is approximately 0.13, indicating that only 13% of the total *Pkd1* transcript is normal. The same ratio was found in the kidneys.

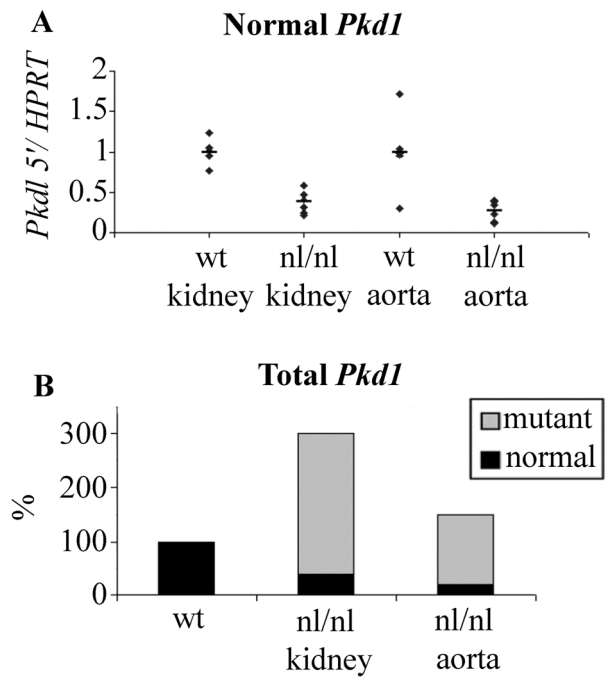


**Figure 1. Quantitative real-time PCR analysis of renal and aortic RNA samples of wild type and *Pkd1*<sup>nl/nl</sup> mice.**

A, Expression of normal *Pkd1* transcript. The 5' *Pkd1* PCR amplifies normal *Pkd1* transcripts. 5' *Pkd1* vs *Hprt* is normalized to the mean ratio of wild-type mice, which has been set to 1. The expression of normal *Pkd1* transcript in the aorta and kidney of *Pkd1*<sup>nl/nl</sup> mice is reduced to 26% and 37%, respectively. B, Total *Pkd1* transcript. The 3' *Pkd1* PCR amplifies both mutant and normal *Pkd1* transcripts. 3' *Pkd1* vs *Hprt* is normalized to the mean ratio of wild type mice, which has been set to 1. The total *Pkd1* transcript is about 1.5 and 2.7 times increased in aorta and kidney of *Pkd1*<sup>nl/nl</sup> mice, respectively. In both kidney and aorta, about 13% of the total transcript is normal.

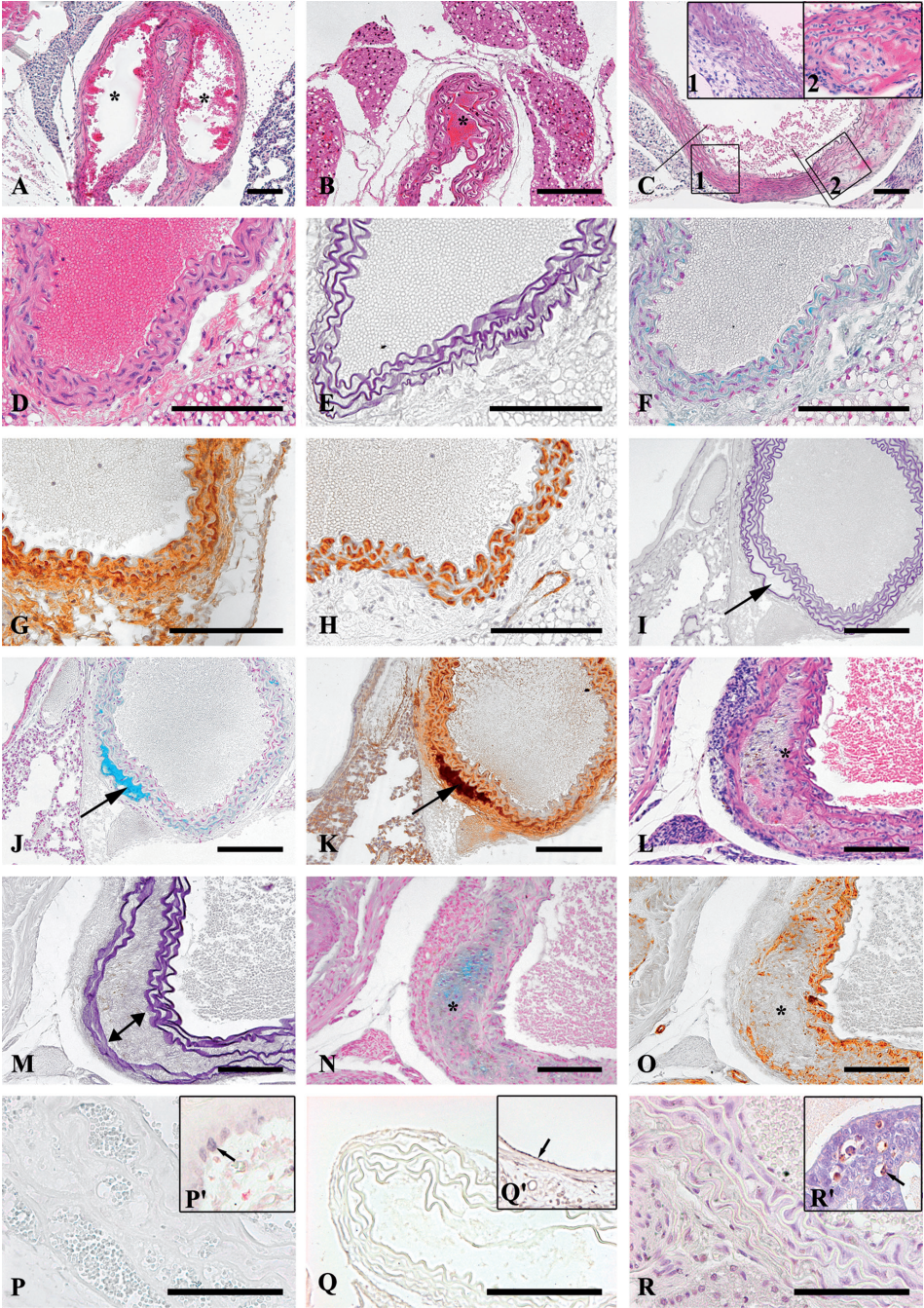


**Figure 2. Dissecting aneurysms in *Pkd1<sup>nl/nl</sup>* mice.** A–C, Hematoxylin Eosin. A, Progressive dissection with 2 pseudo lumens (asterisk) filled with blood. B, Local dissection with intramural bleeding (asterisk). Segment B did not show abnormalities (C1) whereas the surrounding region showed progressive media thickening (C2). D–H, Wild-type aorta: D, Hematoxylin Eosin; E,

Resorcin Fuchsin; F, Alcian blue; G, fibronectin; H, SM-actin. I–K, Sequential sections showing mild medial thickening: I, Resorcin Fuchsin; J, Alcian blue; K, fibronectin. The elastin lamellae are disjoined (arrow; I) showing accumulation of proteoglycans (J) and fibronectin (K) between the elastin lamellae (arrows). L–O, Progressive stages of medial degenerative changes in *Pkd1<sup>nl/nl</sup>* mice: L, Hematoxylin Eosin; M, Resorcin Fuchsin; N, Alcian blue; O, SM-actin. At more progressive regions, media thickening with increased SMCs between the elastin lamellae is found (L), however the elastin lamellae are still intact (M). At these regions proteoglycans are increased (N) whereas SM-actin is decreased (O; asterisks). Ki67 showed no proliferation (P), whereas the positive control (basal cells of the esophagus) did stain positive (arrow; P'). MMP-2 (Q) and TUNEL (R) were both negative in these regions, whereas the positive control was positive, (cystic kidney Q') and (prostate tissue R'), respectively. Bar=100µm.

#### *Characterization of Aortic Pathology in Pkd1<sup>nl/nl</sup> Mice*

Eight of the 9 examined *Pkd1<sup>nl/nl</sup>* mice showed 1 or more abnormalities with different severity at different regions in the descending thoracic and abdominal aorta. Dissections with intramural bleeding were detected in 5 mice. Five mice had progressive dissecting aneurysms showing pseudo lumens all over the descending aorta (3 mice; Figure 2A) or caudal mesenteric artery (2 mice). Two mice showed a local intramural bleeding (Figure 2B). The dissections in the caudal mesenteric arteries were ruptured leading to retro-peritoneal bleeding. One mouse showed local progressive medial thickening in the abdominal aorta without intramural bleeding. The volume of the vessel wall in the areas of these progressive dissections was significantly increased in both the thoracic and abdominal aorta compared with the wild type aortas of the same regions. However, the inner vessel lumen was not changed (Figure 3A and 3B).

The ascending aorta and segment B, which is located between the left carotid and the left subclavia in the aortic arch, were normal. One mouse showed media thickening in the whole area of the aortic arch and descending thoracic aorta except for segment B and the ascending aorta (Figure 2C). In these 2 normal regions SMCs seemed to keep their connection with the matrix, whereas the surrounding region of the aorta showed media thickening with disjoined elastin lamellae.

In addition to dissecting aneurysms, all descending aortas studied contained normal vessel wall structure and incomplete dissections. Medial thickening was found in the thoracic aorta of 6 mice, in the abdominal aorta of 2 mice and intima thickening was found in the thoracic aorta of 1 mouse and in the abdominal aorta of 2 mice. The abnormalities found, were of varying pathological severity. This allowed us to describe the sequential order of events occurring in the media and intima, resulting in a dissecting aneurysm

#### *Media Thickening in Pkd1<sup>nl/nl</sup> Aortas*

The media of the aorta of wild type mice consists of 5 to 6 layers of SMCs alternated with concentric sheets of elastin lamellae (Figure 2D, 2E, and 2H). Surrounding the SMCs extracellular matrix molecules like proteoglycans and fibronectin are present (Figure 2F and 2G). Examination of elastin lamellae in *Pkd1<sup>nl/nl</sup>* aortas by Resorcin Fuchsin staining showed an increase in the space between the lamellae without any fragmentation of the lamellae (Figure 2I). The expression levels of proteoglycans and fibronectin were both notably increased in these regions (Figure 2J and 2K) compared with wild type aortas (Figure 2F and 2G).

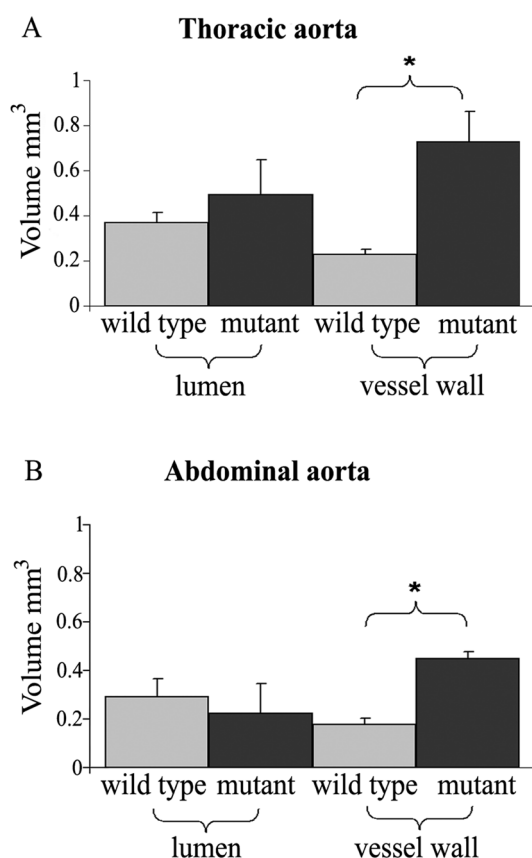


**Figure 3 Volume measurements of aortic lumen and vessel wall in thoracic (A) and abdominal aortas (B).** The volume of vessel wall in both the thoracic and abdominal aortas is increased. The volume of the inner vascular lumens of wild-type mice is not significantly different from mutant mice. \*P=0.05

The proteoglycans versican and perlecan have been shown to be increased in cultures of medial smooth muscle cells from tissue affected by abdominal aortic aneurysm.<sup>15</sup> In our mouse model perlecan was increased whereas versican was absent in both wild type and mutant mice (Table). Using immunohistochemistry no MMP-2 and 9 expression was detected in the wild type or *Pkd1<sup>nl/nl</sup>* aortas (Figure 2Q). Zymography, which is a more sensitive method than immunohistochemistry, revealed none to a very low and similar MMP-2 and MMP-9 expression in both, mutant and wild type, aortas (Figure 4). At other more progressive regions in the aortas, the elastin lamellae were more disjoined with an increased number of smooth muscle cells in between (Figure 2L). However, the elastin lamellae were still intact (Figure 2M).

The SMCs were disarranged with a low SM-actin expression (Figure 2O). Few SMCs had condensed nuclei, however these cells did not stain positive for TUNEL (Figure 2R). Macrophage staining did not reveal any immune-infiltrate in this region (Table). No accumulation of fibronectin was detected in this region. However, proteoglycans were still increased (Figure 2N). The distribution of collagen and fibrillin 1 in the vessel wall of *Pkd1<sup>nl/nl</sup>* mice was comparable to wild type mice. No calcification or expression of osteopontin was detected in all vessels analyzed (Table).

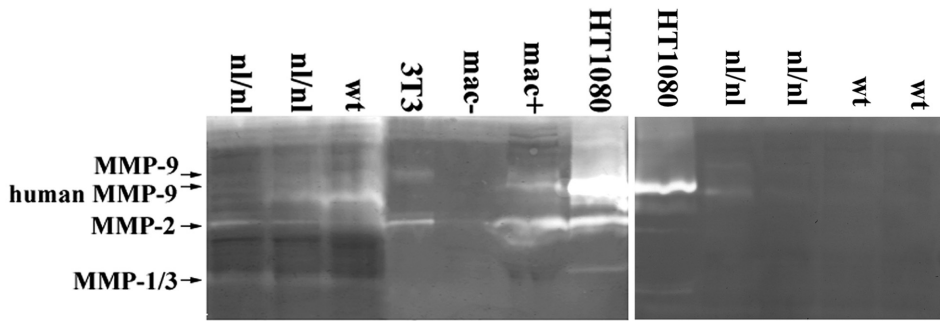


**Figure 4. MMP-2 and 9 expressions in *Pkd1<sup>nl/nl</sup>* and wild type aortas.**

Left panel: *Pkd1<sup>nl/nl</sup>* aortas of mouse 5499 and 5500 (Lane 1 and 2), wild type aorta of mouse 5508 (Lane 3). Positive controls for MMP-2 and MMP-9 expression were NIH 3T3 fibroblast stimulated with 10ng/ml TNF alpha (lane 4) and bone marrow macrophages stimulated with 100ng/ml LPS in serum free medium or with 10% FCS (lane 5 and 6 respectively). A positive control for human MMP-2 and MMP-9 expressions was serum free medium of human fibrosarcoma cell line HT1080 (lane 7). MMP-2 and MMP-9 are indicated by arrows. A lower molecular weight band for MMP-1 and MMP-3 can be observed as well. Right panel: *Pkd1<sup>nl/nl</sup>* aortas of mouse 5510 and 5511 (Lane 2 and 3), wild type aortas of mouse 5514 and 5515 (Lane 4 and 5). Positive control used was serum free medium of human fibrosarcoma cell line HT1080 (Lane 1).

**Table. Immunohistochemical Characterization of *Pkd1<sup>nl/nl</sup>* Mice Compared to Wild Type**

|                    | normal media | media thickening | normal intima | intima thickening |
|--------------------|--------------|------------------|---------------|-------------------|
| SM-alpha-actin     | +            | +/-              | -             | -                 |
| Isolectin          | -            | -                | +             | +/-               |
| Collagen I         | +            | +                | +             | +                 |
| Collagen III       | +            | +                | +             | +                 |
| Collagen           | +            | +                | +             | +                 |
| Fibrillin I        | +            | +                | +             | +                 |
| Elastin            | +            | +                | +             | +                 |
| Fibronectin        | +            | ++               | +             | +                 |
| osteopontin        | -            | -                | -             | -                 |
| calcium deposition | -            | -                | -             | -                 |
| perlecan           | +            | ++               | -             | -                 |
| versican           | -            | -                | -             | -                 |
| proteoglycans      | +            | ++               | -             | +/-               |
| macrophages        | -            | -                | -             | -                 |
| MMP-2              | -            | -                | -             | -                 |
| MMP-9              | -            | -                | -             | -                 |
| Ki-76              | -            | -                | -             | -                 |
| Tunel              | -            | -                | -             | -                 |

\*Elastin lamellae were diverged/broken. - indicates no expression.

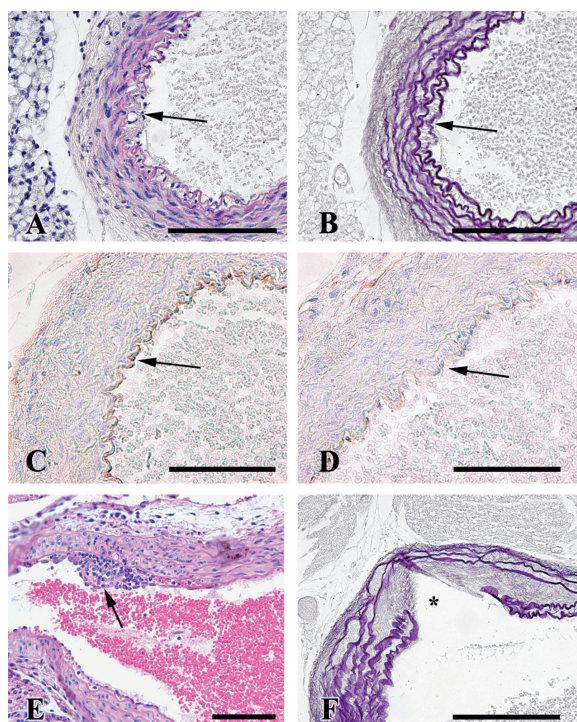


**Figure 5. Histopathologic appearance of the intima in *Pkd1<sup>nl/nl</sup>* mice.**

A–G, Sequential sections showing different stages of intima degenerative changes in *Pkd1<sup>nl/nl</sup>* mice. ECs detach from the internal elastic lamellae (A) and the internal elastic lamina is less compact (B; arrows). At these regions isolectin is decreased (D) compared with control (C; arrows). At more severe regions subendothelial thickening is found (E; arrow). At the entrance of the dissection, the elastin lamellae are broken (F; asterisks). A, E Hematoxylin Eosin; C, D isolectin; B, F Resorcin Fuchsin. Bar=100  $\mu$ m.

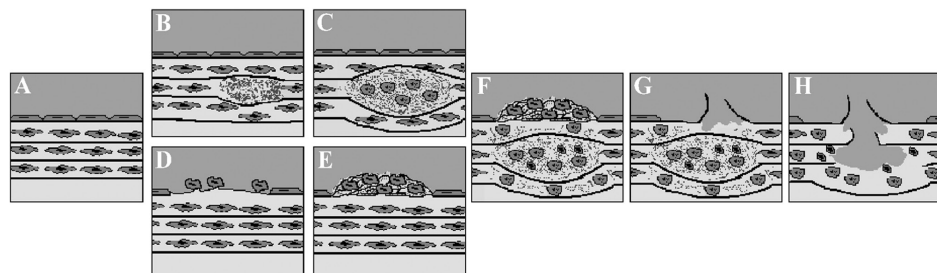


**Figure 6. Model for aneurysm formation in *Pkd1* hypomorphic mice.** A, Normal vessel wall. B, The SMCs produce an excessive amount of matrix components, which accumulate between the elastin lamellae. C, SMCs between the disjoined elastin lamellae proliferate increasing the media thickness. D, The endothelial cells detach from the elastin lamellae. E, The number of intima cells increases and the matrix components accumulate in the subendothelial region. F–H, The combination of a weak aortic media and a tear in the intima leads to (partial) rupture of the vessel wall resulting in intramural bleeding.

#### *Intima Thickening in *Pkd1<sup>nl/nl</sup>* Aortas*

The wild type intima consists of a single layer of flattened endothelial cells on a basal membrane and a subjacent elastin lamellae known as internal elastic lamina. Histological analysis of the intima of *Pkd1<sup>nl/nl</sup>* by Hematoxylin Eosin showed detachment of the ECs from the internal elastic lamina (Figure 5A and B). At these regions isolectin staining was decreased indicating a decreased number of ECs or decreased expression of this marker

on ECs (Figure 5C and D). In other regions the subendothelial region was filled with proteoglycans and the cell numbers were increased (Figure 5E). However, the accumulation of proteoglycans is neither attributable to an increase in versican or perlecan nor did it coincide with increased fibronectin expression as observed in the media thickening (Table). One of the mice showed a tear in the intima next to the degenerative changes within the media resulting into a progressive dissection all over the aorta (Figure 5F). At the entrance of dissection, fragmentation of the elastin lamellae was found, allowing the blood to spread through the aortic media. Despite substantial effort, we could not locate the exact site of rupture in the other animals.

### *Dissecting Aneurysm*

Abnormalities in the media and intima were identified in different regions of the aorta, indicating that the reduction of *Pkd1* in both SMCs and ECs contributes to the formation of dissecting aneurysms by 2 processes, one in the media and the other in the intima. It is not clear whether the initial event of a dissecting aneurysm occurs in the intima or media. We hypothesize that on reducing the expression of polycystin1, the stability of the contact between SMCs and extracellular matrix molecules is reduced. This may disturb some cellular events in the SMCs resulting in a phenotypic change toward a more synthetic phenotype, which means that they produce more matrix components, which accumulate between the elastin lamellae, and start to hyperproliferate (Figure 6B). This results in an increased thickening of the media, which will become weaker (Figure 6C). In the intima the endothelial cells detach from the elastin lamellae (Figure 6D). The number of intima cells increases and the matrix components accumulate in the subendothelial region (Figure 6E). The final event allowing the blood to spread through the aortic media is the tear of the intima (Figure 6F and 6G). When present, next to the degenerative changes within the media, the resistance of the aortic wall to hemodynamic stress reduces, leading to dissection (Figure 6H).

### **Discussion**

The *Pkd1<sup>nl/nl</sup>* mice provided a new model to study aortic dissecting aneurysm formation in ADPKD. It also confirmed the role of *Pkd1* in dissecting aneurysm formation. Our mouse model represents a very severe phenotype of ADPKD with dissections in the aorta, probably due to the fact that the overall *Pkd1* expression in the *Pkd1<sup>nl/nl</sup>* mice is lower than in human ADPKD patients. In patients one wild type *Pkd1* allele is present, whereas in the aortas of these *Pkd1<sup>nl/nl</sup>* mice normal *Pkd1* transcripts is approximately 75% reduced. All dissections were found in the descending aorta whereas the ascending aorta was normal. This is in line with the humane phenotype, as most dissections are found in the descending aorta of ADPKD patients.<sup>3-5</sup> Although intracranial saccular aneurysms are more common in ADPKD than aortic aneurysms, no intracranial aneurysms were found in 3 *Pkd1<sup>nl/nl</sup>* mice at the age of 1 month (data not shown). Different regions of the aorta, indicating that the reduction of *Pkd1* in both SMCs and ECs contributes to the formation of dissecting aneurysms by 2 processes, one in the media and the other in the intima. It is not clear whether the initial event of a dissecting aneurysm occurs in the intima or media.

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phenotype, which means that they produce more matrix components, which accumulate between the elastin lamellae, and start to hyperproliferate. This results in an increased thickening of the media, which will become weaker. In the intima the endothelial cells detach from the elastin lamellae. The number of intima cells increases and the matrix components accumulate in the subendothelial region. The final event allowing the blood to spread through the aortic media is the tear of the intima. When present, next to the degenerative changes within the media, the resistance of the aortic wall to hemodynamic stress reduces, leading to dissection.

Probably, intracranial aneurysms need more time to develop or the method used (immunohistochemistry) is not sensitive enough to detect small saccular aneurysms, or additional factors as elevated blood pressure are essential to develop these kind of aneurysms. Interestingly, in 6- to 9-month-old *Pkd2* mice, Q. Qian *et al* observed macroscopic intracranial vascular complications after induction of hypertension.<sup>16</sup>

A direct correlation between MMP-mediated elastin degradation and elastin calcification has been reported. Perivascular administration of  $\text{CaCl}_2$  to abdominal aortas induced calcification and degradation of elastin fibers.<sup>17</sup> Focal linear calcifications were also detected in a mouse model with reduction of fibrillin-1 showing fragmentation of elastin lamellae and MMP-9 expression.<sup>18</sup> Our data fit into this correlation. In our mouse model MMP-2 and MMP-9 expression was very low, comparable with wild type expression, and no elastin calcification or elastin degradation were found in the media thickening. In addition, we did not observe influx of inflammatory cells which are the main source of MMPs secretion. These properties of dissecting aneurysm formation in *Pkd1<sup>nl/nl</sup>* mice, therefore, seem to be distinct from other models.

Polycystin-1 has been found to be a component of large multiprotein complexes associated with cell-matrix contact at focal adhesions in cultures of renal epithelial cells. It is also associated with adherence junctions and desmosomes in confluent cultures of the renal epithelial cell line Madin-Darby canine kidney (MDCK).<sup>19-21</sup> Polycystin-1 is also implicated in SMC elastin contact. The expression of *Pkd1* is found in dense plaques, which are analogous to focal adhesions in epithelial cells and attach the intracellular contractile filaments of SMCs to the elastin lamellae.<sup>22,23</sup> In our *Pkd1<sup>nl/nl</sup>* mice a widened space between the elastin lamellae was found, suggesting loss of contact between SMCs and elastin or other extra cellular matrix structures. Loss of physical contacts, and hence signals from these interactions, is associated with a phenotypic change toward a more synthetic phenotype of the SMCs.<sup>24</sup> Furthermore, loss of the SMC-elastin interaction may increase SMC proliferation as elastin has a regulatory function controlling SMC proliferation.<sup>25</sup> All these features are found in our *Pkd1<sup>nl/nl</sup>* mouse model, confirming this regulatory mechanism. Although we did not detect ki67-positive cells in the sections available for analysis, cell proliferation should have occurred because increased cell layers were present at certain stages of dissection formation and these cells did not stain for inflammatory cells. The decreased expression of SM-actin and increased expression of proteoglycans and fibronectin in the *Pkd1<sup>nl/nl</sup>* mouse model indicate a response toward a more synthetic phenotype.

Polycystin-1 might be involved in a SMC-matrix adhesion molecule complex, which

maintains a stable SMC phenotype and thereby maintaining the structural vessel wall integrity. Notably, no media thickening was found in segment B of the aortic arch of our mutant mice. The areas adjacent to segment B showed a progressive media thickening, suggesting a normal SMC-matrix contact in this segment. In wild type mice the media of this segment shows characteristics in which it differs from the rest of the aorta. It is negative for SM-actin and has extensive medial innervations. These SMCs have also a different embryonic origin compared with the descending aorta as it is derived from the fourth pharyngeal artery in the embryonic thoracic arterial tree.<sup>14,26</sup> Interestingly, lowering the *Pkd1* expression did not show any influence on the structure of this vessel segment. *Pkd1* expression may not be critical in maintaining the structural vessel wall integrity in the aortic B-segment.

Both polycystin-1 and 2 are probably involved in mechanosensing as they are localized in the primary cilia in renal cells.<sup>27</sup> Polycystin-1 is also concentrated around centrioles and in particles along primary cilia of human umbilical vein endothelial cells (HUVECs).<sup>28</sup> Recently, monocilia were demonstrated on a subset of endothelial cells in low-shear regions.<sup>29</sup> In addition, polycystin-1 has been shown to colocalize with platelet-endothelial cell adhesion molecule-1 (PECAM-1), a cell adhesion molecule also found to be involved in mechanosensing mechanism on the cell membrane.<sup>30,31</sup> PECAM-1 also maintains adherens junction integrity and permeability. The latter function may be influenced by *Pkd1* expression, as *Pkd1* knock-out embryos showed increased microvascular permeability at endothelium cell-cell junctions, edema, and hemorrhages.<sup>10,11</sup> All these factors may weaken the intima and alert the shear stress response, making the intima more prone to rupture.

Thus, lowering of *Pkd1* transcription levels induces degenerative alterations in both the intima and media resulting into a dissecting aneurysm. However, intact regions of the aorta were still present in the *Pkd1*<sup>nl/nl</sup> mice and the overall construction of the aorta with regard to the elastin lamellae is normal. Furthermore, we did not find any abnormalities in these mice at the age of 1 week (data not shown). The elastin assembly may be independent of *Pkd1* expression during development. Matrix components that are involved in elastin organization, like versican, and fibrillin-1, were also not altered compared with the wild-type aortas.<sup>32</sup> This indicates that polycystin1 is probably not implicated in vessel wall development but rather maintains the structural integrity of the vasculature.

## Methods

### *Animals and Tissue Preparation*

The generation of *Pkd1*<sup>nl/nl</sup> mice has been described in detail by Lantinga-van Leeuwen et al.<sup>13</sup> A *loxP*-flanked neomycin selection-cassette was inserted into intron 1 of the *Pkd1* gene by homologous recombination, and a third *loxP* site was inserted in intron 11. Chimeric offspring were backcrossed with C57Bl/6J mice 3 to 6 times. Histological analysis was performed on 9 *Pkd1*<sup>nl/nl</sup> (5 male and 4 female) and 5 (3 male and 2 female) control mice at the age of 1 month and 3 *Pkd1*<sup>nl/nl</sup> (1 male and 2 female) at the age of 1 week. The aortas, from aortic root to the bifurcation of the iliac arteries, were carefully dissected and fixed in 98% ethanol absolute with 2% acetic acid or in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH7.4). In 1 *Pkd1*<sup>nl/nl</sup> mouse only the abdominal aorta was not dissected. Fixed tissues were dehydrated in graded ethanol and xylene and embedded in paraffin. Five-micrometer sections of the complete ascending and descending aorta were completely mounted serially onto glass slides.

### *Antibodies*

SM- $\alpha$ -actin (1A4) 1:2000 (Sigma), Collagen I and III 1:200 (Sanbio), Fibrillin-1 1: 500 (a generous gift from R. Mecham, Washington), Fibronectin 1:4000 (a generous gift from B.N. Bachera, Leiden, The Netherlands), Isolectin 1:20 (Sigma-Aldrich Chemie), Osteopontin 1:100 (a generous gift from L.W. Fisher, Bethesda, Md), Perlecan, Versican alpha and beta 1:20 (a generous gift from J.R. Hassell, Pittsburgh, Pa), Rabbit-anti-mouse Macrophage (A1A 31240) 1:200 (Accurate Chemical and scientific), matrix metalloproteinase (MMP)-2 Ab-4 (75 to 7F7) 1:4 (Calbiochem), MMP-9 11A-5 (11A-5) 1: 100 (Neo Markers), Ki-67 1:3000 (Novocastra), terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) 1:14 (Roche). Secondary antibodies: Rabbit-anti-mouse HRP 1:200, Swine-anti-rabbit HRP 1:200 (DAKO), Goat-anti-rabbit 1:50, Rabbit-peroxidase-antiperoxidase 1:250 (Nordic Immunologic Laboratories), mousebiotinylated (ABC-kit vectastain; Brunschwig Chemie).

#### *Immunohistochemistry*

After deparaffinization, sections were stained using standard procedures.<sup>14</sup> The antibody complexes were visualized using 0.04% diaminobenzidine-tetra-hydrochloride (DAB; Sigma). Antigen retrieval was applied for fibronectin and ki67 staining and for monoclonal antibody 1A4 (SM- $\alpha$ -actin) a complex with the secondary antibody was prepared before incubation. Sections for fibronectin and ki67 staining were incubated in citric acid buffer (0.01 M, pH 6.0) at 98°C for 12 min for antigen-retrieval. The monoclonal 1A4 antibody (SM- $\alpha$ -actin) and secondary antibody rabbit-anti-mouse peroxidase (RAM-PO) were first incubated to form 1A4-RAM-PO complex before adding it on sections following a previously described procedure.<sup>34</sup>

#### *Histochemical staining*

The following histological stainings were performed: Haematoxylin Eosin (HE), Resorcin Fuchsin (RF) for elastin lamellae, Alcian blue for staining glycosaminoglycans and thereby the proteoglycans, von Kossa and alizarin red for calcium deposition and Sirius red for collagen. The serial sections were alternately incubated for the different stainings. All (immuno) histochemical stainings, which were negative in the aortas, showed specific staining in positive control tissue.

Osteopontin stained positive in bronchus epithelial cells, von Kossa stained in trachea cartilage, alizarin red in bone, versican in heart valves, A1A 31240 in inflammatory tissue, Ki67 in basal cells of oesophagus, TUNEL in prostate tissue and MMPs in renal cystic tissue.

#### *RNA Analysis*

Complete aortas and kidneys of 5 control and 6 hypomorphic mice at the age of one month, were dissected and stored in liquid nitrogen until required. Tissues were first homogenized using a magNA lyser (Roche). Total RNA isolation was performed according to the manufacturer's instructions (RNA-bee, AMS biotechnology). RNA was purified with nucleoSpin RNA II columns (Machery-Nagel) and cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen). An aliquot of 100 ng RNA was used to generate cDNA.

Quantitative real-time reverse transcriptase PCR analysis was performed using MyIQ technology (Biorad) and SYBR Green Supermix (Biorad). We developed two *Pkd1* PCR primer pairs: the 5' *Pkd1* PCR specifically amplifies the normal *Pkd1* transcript, and the 3' *Pkd1* PCR amplifies both mutant and normal *Pkd1* transcripts. Primer sequences are described by Lantinga-van Leeuwen et al.<sup>13</sup> Expression of the housekeeping gene *Hprt* (hypoxanthine guanine phosphoribosyl transferase) served as a reference for gene expression. All reactions were performed in triplicate.

#### *Aortic Volume Measurements*

To measure the volumes of aortic walls (including de dissection) and aortic lumens, we used the volume counting method according to Cavalieri.<sup>33</sup> In short, the number of points on a grid hitting the tissue of interest on a series of sections was counted. Volumes could be calculated from the counted numbers by Cavalieri's formula:  $V = \sum[P] \cdot M \cdot a \cdot d$ . In this formula, V is the volume in mm<sup>3</sup>,  $\sum[P]$  is the total number of counted points, M is the magnification, a is the point area in mm<sup>2</sup>, and d is the distance between the counted sections in mm.

#### *Zymography*

Whole aortas of 4 mutant and 3 wild type mice were powdered in liquid nitrogen and a tissue extract was made in 50 mM Tris pH 7.7, 1.5 mM NaCl and 0.25% Triton X100. MMPs expression was analyzed by zymography on gelatin-containing polyacrylamide gels as described.<sup>35</sup> Serum free medium of NIH 3T3 stimulated with 10ng/ml TNF alpha for 24 hours and medium (serum free medium and medium with 10% FCS) of bone marrow macrophages stimulated with 100ng/ml LPS for 24 hours were used as a positive control for mouse MMP-2 and MMP-9 expression. Serum free medium of human fibrosarcoma cell line HT1080 was used for human MMP-2 and MMP-9.

### Statistics

The aortic volumes of the wild type and mutant mice were compared with an independent sample 2-tailed T-test. A P value of <0.05 (2 tailed) was considered significant. All analyses were performed with the Statistical Package for Social Studies version 11.0 (SPSS Inc, Chicago, Ill).

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# Chapter 5

## **Vascular smooth muscle cells specific *Pkd1*-inactivation and adaptation to hypertension.**

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Lab Invest. 2011 Jan;91(1):24-32.

## Abstract

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a multi-system disorder characterized by renal, hepatic and pancreatic cyst formation and cardiovascular complications. The condition is caused by mutations in the *PKD1* or *PKD2* gene. In mice with reduced expression of *Pkd1* dissecting aneurysms with prominent media thickening have been seen. To study the effect of selective disruption of *Pkd1* in vascular smooth muscle cells (SMCs), we have generated mice in which a floxed part of the *Pkd1* gene was deleted by *Cre* under the control of the *SM22* promotor (*SM22-Pkd1<sup>del/del</sup>* mice). *Cre* activity was confirmed by X-gal staining using lacZ expressing *Cre* reporter mice (R26R), and quantitative polymerase chain reaction indicated that in the aorta *Pkd1* gene expression was strongly reduced while *Pkd2* levels remained unaltered.

Histopathological analysis revealed cyst formation in pancreas, liver and kidneys as the result of extra-vascular *Cre* activity in pancreatic ducts, bile ducts and in the glomerular Bowman's capsule. Remarkably, we did not find any spontaneous gross structural blood vessel abnormalities in mice with somatic *Pkd1* gene disruption in SMCs or simultaneous disruption of *Pkd1* in SMCs and endothelial cells (ECs). Extensive isometric myographic analysis of the aorta did not reveal differences in response to KCl, acetylcholine, phenylephrin or serotonin, except for a significant increase in contractility induced by phenylephrin on arteries from 40 weeks old *Pkd1<sup>del/+</sup>* germ-line mice. However, *SM22-Pkd1<sup>del/del</sup>* mice significantly showed reduced decrease in heart rate upon Angiotensin II-induced hypertension. The present findings further demonstrate in vivo, that adaptation to hypertension is altered in *SM22-Pkd1<sup>del/del</sup>* mice.

## Introduction

Autosomal Dominant Polycystic Disease (ADPKD) is one of the most common inherited diseases, affecting 1:1000 individuals worldwide. The disease is characterized by the progressive kidney enlargement and destruction of the normal renal tissue by many fluid-filled cysts, and progresses to end-stage renal failure (ESRF) in most patients. However, it is a slowly progressive disease with ESRF usually around the age of sixty.<sup>1</sup>

Approximately 85% of cases are due to *PKD1* mutations and in 15% the disease is caused by a mutation in *PKD2*.<sup>2</sup> Both *PKD1* and *PKD2* are expressed in a variety of cell-types and tissues including renal epithelium, hepatic bile ducts, pancreatic ducts, vascular smooth muscle cells (SMCs), and endothelial cells (ECs).<sup>3-5</sup> ADPKD is also associated with cysts in the liver and pancreas as well as cardiovascular complications such as hypertension and aneurysms.<sup>6</sup>

Hypertension is very common in ADPKD, and occurs in the majority of patients before any substantial reduction in renal function is observed. Furthermore, hypertension occurs at a much earlier age in patients with ADPKD than in the general population.

Hypertension is also associated with a rapid progression towards renal failure and increased cardiovascular complications (for review see<sup>7</sup>). The prevalence of cerebral aneurysms in ADPKD patients is approximately 10-fold higher than in the general population and about 27% among ADPKD patients with a family history for aneurysms.<sup>8,9</sup>

Several mouse models have been established to study ADPKD using targeted disruption of the *Pkd1* or *Pkd2* gene. Homozygous *Pkd1* and *Pkd2* knock-out mice die in utero, around embryonic day 15, due to severe cystic disease, vascular defects and/or abnormalities of the placental labyrinth, while heterozygous *Pkd1* knock-out mice showed

minimal renal cyst formation in adulthood.<sup>4,10-15</sup> Furthermore, we described a *Pkd1* mouse (hypomorphic) model, with low expression of *Pkd1* showing progressive polycystic kidney disease and aortic dissecting aneurysms, indicating that *Pkd1* is implicated in the structural integrity and function of the vasculature.<sup>16</sup>

In the hypomorphic *Pkd1<sup>nl/nl</sup>* mice a very prominent media thickening was seen in most of the animals analyzed. Since smooth muscle cells is the most abundant cell type in the media, we set out to study the effect of *Pkd1* gene disruption in vascular smooth muscle cells on blood vessel function. To this aim we generated mutant mouse lines with a targeted disruption of the *Pkd1* gene in SMCs, by crossing mice containing floxed *Pkd1* alleles with *SM22Cre*. We previously showed that *Pkd1* gene disruption in vascular SMCs results in a reduced myogenic tone.<sup>18</sup> Functional roles for the polycystins in mechanosensation have been proposed in ECs as well as SMCs. In endothelial cells, the proteins are involved in fluid shear stress sensing, thereby regulating calcium signalling and nitric oxide release. This affects vasodilatation in response to increased blood flow.<sup>19</sup> In SMCs, the polycystins regulate the activity of the stretch-activated cation channels (SAC). In mice with reduced SMCs *Pkd1* expression, SAC activity was decreased and the threshold pressure for myogenic contraction was significantly shifted to higher pressure values.<sup>18</sup> The ratio of polycystin1/polycystin2 regulates SACs activity since polycystin-2 inhibits channel opening, while polycystin1 reverses this inhibition.<sup>18</sup> It is likely that alterations in intracellular  $\text{Ca}^{2+}$  homeostasis contribute to the vascular phenotype in PKD.<sup>20,21</sup> Here we show that disruption of the *Pkd1* gene in these cells did not induce gross structural abnormalities in blood vessels, even not when *Pkd1* was disrupted in endothelial cells, as well. However, an increase in blood pressure in *SM22-Pkd1<sup>del/del</sup>* mice, induced by Angiotensin II treatment, resulted in reduced heart rate adaptation.

## Results

*Inactivation of Pkd1 in smooth muscle and endothelial cells does not result in gross structural blood vessel abnormalities.*

To inactivate *Pkd1* in SMCs, we crossed *Pkd1<sup>del/lox</sup>* mice with *SM22Cre* mice to yield *SM22-Pkd1<sup>del/del</sup>*. We carefully analyzed aortas of 6 *SM22-Pkd1<sup>del/del</sup>* mice (age 6 months) with *Pkd1* gene disruption in SMCs but did not see any abnormalities (Figure 1A-C). Since the matrix metalloproteinase (MMP)-2 and -9, are key-activators in blood vessel remodeling we performed zymography to analyse MMP-2 and MMP-9 activity. No differences were observed between the different genotypes (not shown). Subsequently, we inactivated *Pkd1* in both SMCs and ECs by crossing *SM22-Pkd1<sup>del/del</sup>* with *Tie2-Pkd1<sup>del/del</sup>* mice to yield *SM22;Tie2-Pkd1<sup>del/del</sup>* mice. We carefully analyzed aortas of 4 *SM22;Tie2-Pkd1<sup>del/del</sup>* mice (age 6 months). Also these aortas were morphologically normal. As combined inactivation of *Pkd1* in SMCs and ECs did not show any vascular phenotype, we did not further analyze the aortas of *Tie2-Pkd1<sup>del/del</sup>* mice with *Cre* activity only in ECs.

*Inactivation of Pkd1 in SM22Cre expressing cells induces cysts in pancreas, liver and kidneys.*

All neonatal *SM22-Pkd1<sup>del/del</sup>* mice (age 3 and 4 days, n=7) showed a few large and small pancreatic cysts (Figure 1F) as well as dilated glomeruli in kidneys. The liver showed no cysts at this age. Most mice survived and were sacrificed at the age of 6 months, (n=10) large cysts were found in pancreas, liver and kidneys (Figure 1G-I). Cysts in pancreas

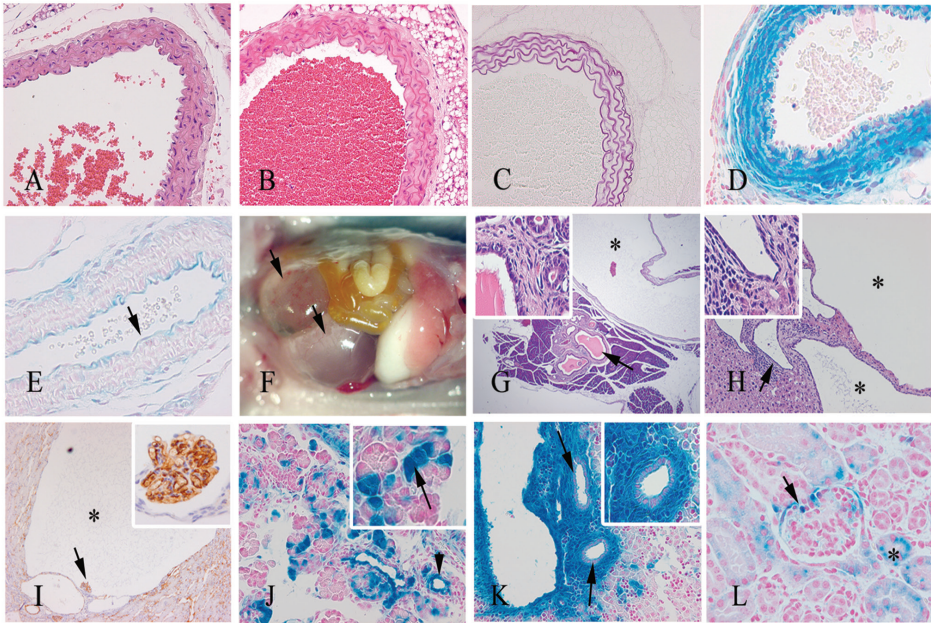


**Figure 1. Blood vessel structure and cyst formation in *SM22-Pkd1<sup>del/del</sup>* mice; the activity of *Cre* in *SM22Cre* and *Tie2Cre* mice.**

(A) Wild type aorta, HE staining. (B) *SM22-Pkd1<sup>del/del</sup>* aorta, HE staining. (C) *SM22-Pkd1<sup>del/del</sup>* aorta, elastin lammellae staining. (D) Cre induced LacZ activity in SMCs in aorta of 3-days-old *SM22Cre;R26R* mouse. (blue staining) (E) *Tie2Cre* induced LacZ activity in ECs (arrow) in aorta of 3-days-old *Tie2Cre;R26R* mouse (blue staining) (F-I) Organs of *SM22-Pkd1<sup>del/del</sup>* mice (F) Large pancreatic cysts (arrows) in the abdomen of 3-days-old *SM22-Pkd1<sup>del/del</sup>* mouse (G) Small pancreatic cysts (arrow) and large cysts (asterisk). (H) Small (arrow) and large cysts (asterisk) in liver. Insets in G and H show fibrotic tissue around cysts. (I) Glomerular cysts (asterisk). Inset shows CD31 staining in glomerulus. (J-L) Recombination of lacZ (blue staining) under control of *SM22* promoter in different organs of 3-days-old *SM22Cre;R26R* mice. (J) lacZ expression in pancreatic ducts (arrowhead). Inset show some acini cells express lacZ (arrow). (K) *SM22Cre* activity in liver. Bile ducts express lacZ (arrow and inset). (L) *SM22Cre* activity in kidney. The parietal layer of Bowman's capsule of mature glomeruli express lacZ (arrow). Cortical tubules show nonspecific staining (asterisk), which is also observed in control R26R mice (not shown).

and liver were surrounded by spindle-like cells which were positive for  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA, not shown) indicating infiltration of myofibroblasts (Figure 1, insets in G and H). The cysts were localized in groups, and few cysts were hyperplastic. In the pancreas, the cystic phenotype was more progressive compared to kidneys and liver, with larger numbers and size of cysts. Cysts in the pancreas and liver seem to originate from the pancreatic ducts and bile ducts, respectively, while no loss of pancreatic acini or hepatocytes in liver were detected.

In the kidneys, a few dilated glomeruli, 1-6 dilatations per section, were found in neonates at day 3. In 6 months old mice, large cysts were found and many of these cysts contained the glomerular tuft, which stained for the endothelial marker CD31, indicating

the presence of glomerulus-derived-cysts (Figure 1I). Not all glomeruli were dilated and kidneys showed many normal glomeruli as well. None of the cysts stained positive for the nephron-segment-specific markers (megalin for proximal tubules, Tam Horsfall protein for distal tubules and loops of Henle and aquaporin-2 for collecting ducts). We also did not find dilatations of lymphatic or blood vessels, as determined by lyve-1, CD31 and  $\alpha$ -SMA staining.

*Tie2-Pkd1<sup>del/del</sup>* mice (n=13) did not show any abnormalities in the liver, kidneys, and pancreas at the age of 7, 8, 10, and 12 months. Only 2 mice showed 1 cyst in the liver (age 7 and 10 months). The *SM22;Tie2-Pkd1<sup>del/del</sup>* mice with Cre activity in both SMCs and ECs (n=7) showed the same phenotype as the *SM22-Pkd1<sup>del/del</sup>* mice. Taken together, these results indicate that the *Tie2-Pkd1<sup>del/del</sup>* genotype does not cause gross structural abnormalities while the *SM22-Pkd1<sup>del/del</sup>* genotype is responsible for the cysts formation in the different organs of the *SM22-Pkd1<sup>del/del</sup>* and *SM22;Tie2-Pkd1<sup>del/del</sup>* mice.

#### *Cre Activity in aortas of SM22Cre and Tie2Cre mice.*

To monitor the activity of Cre in the *SM22Cre<sup>+</sup>* and *Tie2Cre<sup>+</sup>* mice, we crossed these mice with a LacZ expressing Cre reporter mouse line, *R26R*.<sup>23</sup> X-gal staining of *SM22Cre;R26R* (age 3 days, n=2) aortas, revealed that efficient recombination of the reporter construct had occurred in SMCs in the media and the adventitia, resulting in expression of the LacZ-gene (Figure 1D). In *Tie2Cre;R26R* (age 3 days, n=1), LacZ expression was found in most ECs in the intima (Figure 1E). *Pkd1* gene disruption by Cre was confirmed by PCR analysis of the DNA derived from the tails of mice carrying the *Pkd1<sup>lox/+</sup>* alleles as well as *SM22Cre* or *Tie2Cre* (Figure 2B). Even more, quantitative PCR revealed a strong reduction in *Pkd1* mRNA levels in aorta's of *SM22-Pkd1<sup>del/del</sup>* mice compared to *SM22-Pkd1<sup>del/+</sup>* and controls, while *Pkd2* mRNA levels remained unaltered (Figure 2A).

#### *Cre Activity in pancreas, liver and kidneys of SM22Cre and Tie2Cre Mice.*

To explain the phenotype of the *SM22-Pkd1<sup>del/del</sup>* mice, we analyzed Cre activity in the kidneys, liver, and pancreas in *SM22Cre;R26R* mice (age 3 days). X-gal staining was found in all three organs and it matched with the origin of cyst formation found in these organs in *SM22-Pkd1<sup>del/del</sup>* mice. In the pancreas, many serous acini and the ducts were positive for lacZ expression (Figure 1J). In the liver, expression was found in most bile ducts and in mesenchymal cells around the ducts (Figure 1K). In addition, blood cell precursors e.g. myeloblasts, myelocytes, granulocytes, and megakaryocytes were also positive for lacZ. Hepatocytes did not show lacZ expression. The kidneys showed lacZ expression in the parietal layer of Bowman's capsule of mature glomeruli (Figure 1L). The control *R26R* mice, without the Cre transgene, did not show X-gal staining, except for a subset of proximal tubules. This was also found in the renal cortical tubules of *SM22Cre;R26R* and *Tie2Cre;R26R*, indicating nonspecific signal (Figure 1L). Smooth muscle cells of blood vessels in all organs were positive for X-gal.

Deletion of *Pkd1* under the control of *Tie2* promotor did not result in a phenotype. *Tie2Cre;R26R* reporter mice, neonatal age 3 days, showed lacZ expression in endothelial cells in large vessels (Figure 1E), in capillaries, and in blood cell precursors (not shown), which is in line with previous reports.<sup>28</sup>

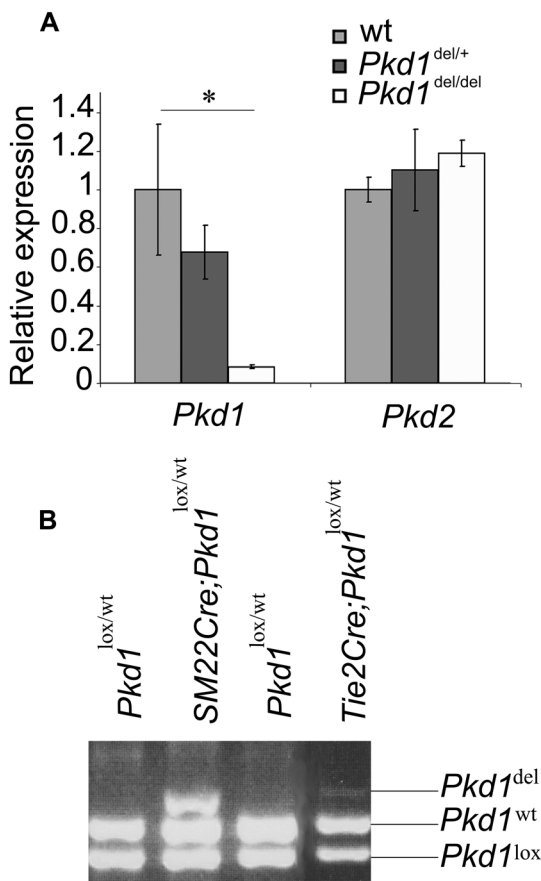


**Figure 2. SM22Cre and Tie2Cre mediated recombination of the floxed *Pkd1* allele.**

A) *Pkd1* and *Pkd2* mRNA levels in aorta's from SM22-*Pkd1*<sup>del/del</sup> (n=13), SM22-*Pkd1*<sup>del/+</sup> (n=9) or control (n=31) mice. Expression was normalized to the housekeeping gene. Results are presented as mean  $\pm$  SD of expression relative to the expression of controls. B) Agarose gel analysis of PCR products amplified from genomic DNA isolated from tails of adult SM22-*Pkd1*<sup>lox/wt</sup>, Tie2-*Pkd1*<sup>lox/wt</sup> and *Pkd1*<sup>lox/wt</sup>. Three bands can be seen for both Tie2-*Pkd1*<sup>lox/wt</sup> and SM22-*Pkd1*<sup>lox/wt</sup> mice; the wild type *Pkd1* allele (*Pkd1*<sup>wt</sup>), the floxed *Pkd1* allele (*Pkd1*<sup>lox</sup>) and the partially deleted *Pkd1* (*Pkd1*<sup>del</sup>) indicating that floxed *Pkd1* allele is deleted upon Cre expression in both mouse models. The intensity of the 366-bp deletion-specific PCR product is relatively weak compared to the floxed or wild-type alleles since Cre-mediated recombination occurred only in SMCs or ECs respectively. In control mice, without Cre, the deletion-specific product was absent.

### Selective inactivation of *Pkd1* in smooth muscle cells does not affect contractility

To analyze whether *Pkd1* gene deletion has more subtle effects on vascular function, several series of isometric myographic analyses were performed on aortas. First, heterozygous SM22-*Pkd1*<sup>del/+</sup> mice and controls as well as homozygous SM22-*Pkd1*<sup>del/del</sup> mice and their controls were analyzed at 11 weeks of age (Figures 3A-C). At this age the IC90 (internal diameter at 90 mm Hg pressure) is small, around 415  $\mu$ m, and vasoconstriction induced by phenylephrin is low (0.2 mN/mm at 10<sup>-5</sup> M)(Figure 3C). No differences were observed between the different genotypes (Figure 3C). We also found no differences between IC90 at vasoconstriction obtained with passive depolarization of the vessels with 80 mM KCl (PSS 80K), between activation curves obtained with serotonin or the vasoconstrictor U46619, and when looking at dilatation obtained with acetylcholine (Figures 3A, 3B).

Next series of measurements were performed on the heterozygous SM22-*Pkd1*<sup>del/+</sup> mice and their controls, as well as the germ-line *Pkd1*<sup>del/+</sup> (which carry a deletion allele in all cells) and wild-type controls, both performed on older mice, aged 40 weeks (Figures 3D-I). The 40-weeks-old homozygous mutants SM22-*Pkd1*<sup>del/del</sup> were not available because of the cystic phenotype (see above). No differences were found between the IC90, with

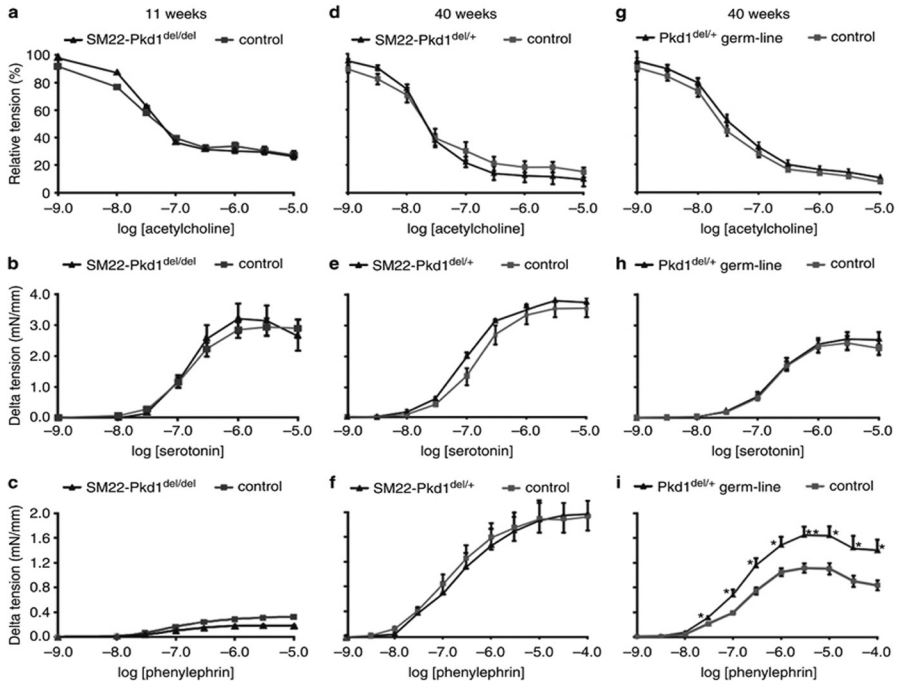


**Figure 3: Myographic analysis on aorta from 11 weeks and 40 weeks-old mice.** (A, D, G) Dose-effect curve of the variation in tension, relative to precontracted tension, induced by different concentrations of acetylcholine. Doses were added cumulatively after a precontraction with 1  $\mu$ M phenylephrin. Experiments were carried out on 11 weeks-old mice *SM22-Pkd1<sup>del/del</sup>* (n=22) and their controls (*SM22Cre* and *Pkd1<sup>lox/lox</sup>*, n=55)(A), and on 40 weeks-old *SM22-Pkd1<sup>del/+</sup>* (n=10) and their controls (*SM22Cre* and *Pkd1<sup>lox/+</sup>*, n=16)(D), as well as with *Pkd1<sup>del/+</sup>* germ-line mice (n=17) and their controls (wild type, n=12)(G). (B, E, H) Dose-effect curve of the variation in tension, relatively to resting tension, induced by various concentrations of serotonin. Doses were added cumulatively and experiments were carried out on the same mice as in A, D and G. (C, F, I) Dose-effect curve of the variation in tension, relatively to resting tension, induced by various concentrations of phenylephrin. Doses were added cumulatively and experiments were carried out on the same mice as in A, D, G.

1029  $\pm$  16  $\mu$ m and 1053  $\pm$  9  $\mu$ m for *SM22-Pkd1<sup>del/+</sup>* (n=10) and their controls (n=16) respectively (p=0.49), and with 967  $\pm$  10  $\mu$ m and 979  $\pm$  10  $\mu$ m for *Pkd1<sup>del/+</sup>* (n=17) and their control wild types (n=12) respectively (p=0.79). The vasodilatation induced by acetylcholine was not significantly different in both series of experiments (Figures 3D, 3G). The vasoconstriction induced by depolarization with 80 mM KCl was not different in both series (not shown). No difference was observed neither on the vasoconstriction induced by serotonin (Figures 3E, 3H), nor on the one obtained with U46619 (not shown). The vasoconstriction induced by phenylephrin was not different between smooth muscle specific heterozygous *SM22-Pkd1<sup>del/+</sup>* and their controls (Figure 3F). Interestingly, they were significantly higher in the germ-line *Pkd1<sup>del/+</sup>* mice compared to wild-type (p=0.007 at 3  $\mu$ M), as previously reported by Morel et al (2009) for 30 weeks old mice (Figure 3I).<sup>17</sup>

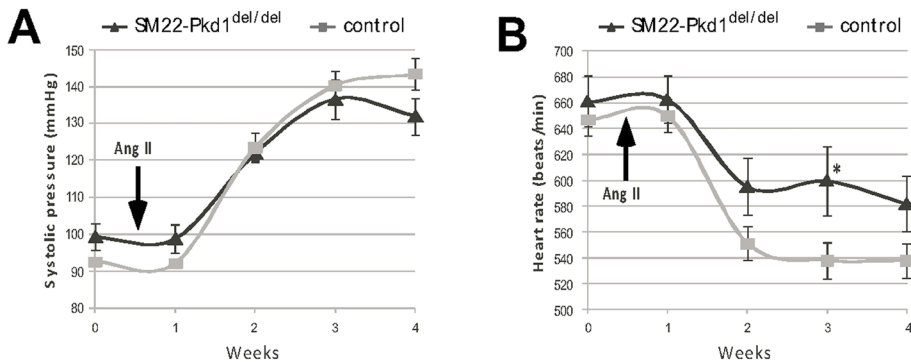


**Figure 4:** (A) Systolic blood pressure and (B) heart rate measured in *SM22-Pkd1<sup>del/del</sup>* (n=6) and their controls (*SM22Cre* mice and *Pkd1<sup>lox/lox</sup>* littermates, n=18) at 1 to 4 weeks of angiotensin II infusion. Mean  $\pm$  SEM are shown. Difference in heart rate is significant at week 3 (p=0.041). The five measurements during each week were averaged. The pumps containing AngII were implanted between week 0 and week 1.

#### *Increased blood pressure induces mild media thickening and impaired adjustment of heart rate in SM22-Pkd1<sup>del/del</sup> mice*

Since the hypomorphic *Pkd1<sup>nl/nl</sup>* mice show a strong renal cystic phenotype, it is anticipated that those mice have an elevated blood pressure which could contribute to the vascular pathology. As baseline arterial pressure was not altered in *SM22-Pkd1<sup>del/del</sup>* mice compared to controls.<sup>18</sup> We induced high blood pressure by infusion of Angiotensin II (AngII)(Figure 4). Histological analysis did not reveal structural differences between Angiotensin II-treated *SM22-Pkd1<sup>del/del</sup>* mice and controls (not shown). Interestingly, AngII-induced decrease in heart rate was significantly less pronounced in *SM22-Pkd1<sup>del/del</sup>* (p = 0.041, at 3 weeks), although the systolic pressure response was not different (Figures 4A, 4B).

#### **Discussion**

Previously, we analyzed a *Pkd1* hypomorphic mouse model, *Pkd1<sup>nl/nl</sup>*, with reduced levels of *Pkd1* transcripts in the aortas and kidneys.<sup>16</sup> These mice showed dissecting aneurysm formation with very prominent abnormalities in the media and mild intima involvement. In addition, they have severe polycystic kidney disease.

In the current study, we generated mouse models to analyze the consequences of the specific deletion of *Pkd1* in SMCs, which are the major component of the media. These mice, however, did not show any structural alterations of the aortic vessel wall in mice analyzed up to 6 months. Moreover, disruption of *Pkd1* simultaneously in SMCs and ECs did not result in structural blood vessel alterations, even though the recombination levels were high in blood vessels as shown by the Cre reporter and by strongly reduced *Pkd1* mRNA levels. This is in agreement with early expression of the endogenous *SM22* and *Tie2* genes, around embryonic day 8.<sup>29,30</sup> Our data indicate that *Pkd1* gene disruption in SMCs alone and/or in ECs is not sufficient to induce gross structural abnormalities in the

aorta, suggesting that additional triggers are needed to induce or to accelerate the phenotype. For the renal cystic phenotype we previously showed that renal injury is a trigger that strongly accelerates polycystic kidney disease.<sup>31</sup>

Extensive isometric myographic analysis did also reveal that no difference in response to KCl, acetylcholine, phenylephrin, U46619 or serotonin was observed on young mice (11 weeks) or on older mice (40 weeks), on heterozygous or on homozygous animals with a selective disruption of *Pkd1* in smooth muscle cells. Only the 40 weeks old heterozygous *Pkd1*<sup>del/+</sup> mice, which carry a deletion allele in all cells, show a significant increase in vascular contractility in response to phenylephrin. This increase has been previously reported in the same mutants aged 30 weeks, whereas at 12 and 20 weeks no differences were observed.<sup>17</sup> Our results suggest that heterozygous *Pkd1* gene disruption in vascular SMCs is not sufficient to explain the increased phenylephrin response in *Pkd1*<sup>del/+</sup> mice and that other cell types probably contribute to this vascular phenotype. In addition, the effect is building up over time.

Importantly, although we did not observe spontaneous structural alterations in the aorta's of *SM22-Pkd1*<sup>del/del</sup> mice, these mice showed a decreased myogenic tone in resistance arteries.<sup>18</sup> Interestingly, the ratio of polycystin-1/polycystin-2, which is disturbed in the deletion mutants, is of crucial importance for the regulation of stretch-activated ion channels (SACs) in arterial myocytes.<sup>18</sup> Activation of SACs regulates myocyte contraction and thereby the vascular tone. When we implanted the angiotensin II releasing pumps to induce hypertension, the heart rate significantly slowed down in all genotypes. This decrease in heart rate is the result of adaptation of the cardiac outflow to increased peripheral resistance and/or to the central nervous system effect of angiotensin.<sup>32,33</sup> Interestingly, in the homozygous *SM22-Pkd1*<sup>del/del</sup> mice, this decrease in heart rate is significantly less prominent compared to the control mice. Cardiac output (stroke volume x heart rate) is known to drop upon an increase in blood pressure, as for instance induced by angiotensin II infusion.<sup>34</sup> However, according to the classical Guyton model (Guyton et al, 1972), without autoregulation (i.e. myogenic tone), cardiac output in response to hypertension is predicted to remain high.<sup>32,34</sup> This model fits nicely with our data as anticipated by a loss of myogenic tone in *SM22-Pkd1*<sup>del/del</sup> mice.<sup>18</sup> Indeed, in these mice, the decrease in heart rate (i.e. cardiac output) is significantly less prominent compared to the control mice. Another possible mechanism of the hypertension-induced bradycardia may include a central effect through AT1 receptors in the central nervous system.<sup>33</sup>

Several other explanations could be given why without additional triggers the hypomorphic *Pkd1*<sup>nl/nl</sup> mice, and also *Pkd1* knock-out mice, show structural abnormalities of the blood vessels at neonatal or embryonic stages respectively, while the *SM22;Tie2-Pkd1*<sup>del/del</sup> mice do not.<sup>4,10;11;16</sup> The genetic background may affect the process of blood vessel remodeling, since the complex mouse models used in this study consisted of a mixture of different genetic backgrounds. Furthermore, low *Pkd1*-expression in other cell types than SMCs and ECs in which *Pkd1* is not disrupted in the *SM22;Tie2-Pkd1*<sup>del/del</sup> mice (for instance immune cells), may contribute to the process of aneurysm formation in *Pkd1*<sup>nl/nl</sup> mice, thereby accelerating the process. Therefore, it will be interesting to study the effects of increased blood pressure in *SM22;Tie2-Pkd1*<sup>del/del</sup> mice on full C57BL/6 background, in future experiments.

Although no spontaneous structural abnormalities of the vasculature were detected, we observed cyst formation in pancreas, liver, and kidneys when the *Pkd1* gene was deleted under the control of *SM22* promotor. Expression of LacZ matched with the sites of cyst formation and was found in pancreatic ducts and hepatic bile ducts, which are the origin of the cysts. In the kidneys, only glomerular cysts were found and recombination did occur in the parietal layer of Bowman's capsule of glomeruli. It is known that *Pkd1* is also expressed in pancreatic ducts, hepatic bile ducts, and Bowman's capsule and thus coincides with the origin of the cysts as well.<sup>5;35;36</sup> Also in ADPKD patients these cysts can be found. Our data indicate that the *SM22* promoter fragment that regulates the expression of *Cre* is not exclusively expressed in vascular smooth muscle cells. Expression of the promoter in specific mesenchymal cells during development may explain the observed expression pattern.<sup>37</sup>

Despite the presence of large cysts, the function of the pancreas was not severely affected, as no loss of acini was found. In the liver, no loss of hepatocytes was noticed either. Furthermore, the mutant kidneys showed many normal glomeruli beside the dilated ones. These results may explain why the mutant mice were able to survive for several months even with the presence of large cysts especially in the pancreas.

The renal glomerular cystic phenotype was relatively mild and did not affect blood pressure. Glomerular cysts can also be found as part of ADPKD, predominantly in fetal kidneys but also in adult end-stage kidneys in addition to tubular cysts.<sup>38;39</sup> In addition, glomerular cysts can develop as distinct entity, Glomerulocystic kidney disease (GCKD), or in the context of several other renal diseases.<sup>40-42</sup> Also in *Pkd1*-mutant mouse models glomerular cysts have been observed. These cysts were only observed in *Pkd1* knock-out mice but not in hypomorphic mice that have low levels of *Pkd1* gene expression, suggesting that total absence of *Pkd1* is necessary to induce the formation of glomerular cysts.<sup>10;11;15;17;43;44</sup> How these cysts arise is not entirely clear. Since *Pkd1* is deleted in Bowman's capsule, the suggested mechanism that urinary tract infections lead to increased pressure in Bowman's space seems not very likely in *SM22-Pkd1<sup>del/del</sup>* mice.<sup>45</sup> Defects in cilia signaling, cellular signaling, cell-cell and cell-matrix interactions, which have been observed in ADPKD tubular cysts, probably play a more important role. In addition, as polycystin-1 is known to affect migration and proliferation,<sup>46;47</sup> the absence of *Pkd1* might disturb the recruitment of podocytes from the glomerular parietal epithelial cells, which proliferate and migrate into the glomerular tuft and differentiate into podocytes.<sup>48</sup>

In conclusion, targeted deletion of *Pkd1* in SMCs does not induce major structural blood vessel abnormalities, spontaneously, in mice. Increased blood pressure, however, revealed a mildly reduced adaptation of the heart-rate. It remains to be determined whether the decrease in myogenic tone observed in these mice is involved in this phenotype.<sup>18</sup>

## Method

### Mice

The generation of *Pkd1<sup>del2-11</sup>* and *Pkd1<sup>lox2-11</sup>* alleles has been described previously.<sup>17;22</sup> The *Pkd1<sup>lox2-11</sup>* (*Pkd1<sup>lox</sup>*) allele contains two *loxP* sites inserted into intron 1 and 11. In the presence of the DNA recombinase *Cre* the *loxP* flanked sequence was deleted to form the *Pkd1<sup>del2-11</sup>* (*Pkd1<sup>del</sup>*) allele. The floxed *Pkd1* mice were crossed with *SM22Cre* mice (*Tgln-Cre*, Jackson laboratories, Bar Harbor, USA) to target smooth muscle cells (SMCs)

or with Tie2Cre mice (kindly provided by Dr. B. Arnold, Heidelberg, Germany) to target endothelial cells (ECs). This yielded *SM22Cre<sup>+</sup>-Pkd1<sup>del/del</sup>* mice (*SM22-Pkd1<sup>del/del</sup>*) and *Tie2Cre<sup>+</sup>-Pkd1<sup>del/del</sup>* mice (*Tie2-Pkd1<sup>del/del</sup>*). To generate *SM22Cre<sup>+</sup>;Tie2Cre<sup>+</sup>-Pkd1<sup>del/del</sup>* mice (*SM22;Tie2-Pkd1<sup>del/del</sup>*), *SM22-Pkd1<sup>del/del</sup>* mice were crossed with *Tie2-Pkd1<sup>del/del</sup>* mice. Littermates that were homozygous or heterozygous for the floxed *Pkd1* gene and mice only expressing the Cre transgene, were used as controls. To analyze Cre activity in vivo, *Tie2Cre* and *SM22Cre* mice were crossed with the *ROSA26 (R26R)* LacZ-expressing Cre reporter mice.<sup>23</sup> All experiments using mice were approved according to the Dutch and French law and conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Approval was granted by a local ethics review board.

#### *PCR for Pkd1 gene disruption in ECs and vascular SMCs*

Genotypes were assessed by PCR analysis of tail genomic DNA using a reverse primer in intron 11 combined with a forward primer in intron 1 (del allele) or a forward primer in intron 11 (floxed and wild-type alleles) as described previously for genotyping of strains.<sup>22</sup>

#### *Real-time quantitative PCR*

Quantitative PCR was performed on mRNA isolated from aortas of 30 weeks old *SM22-Pkd1<sup>del/del</sup>*, *SM22-Pkd1<sup>+/-</sup>* and control mice. For quantitative PCR (qPCR) analysis, primer sequences are as follow: <sup>Pkd1</sup>: Forward: GCC ATC CAG CAC TTC CTA GT, Reverse: GAG AAG CCG ATC CAC ACA TC; <sup>Pkd2</sup>: Forward: AGGTGTTAG-GACGGCTGCT, Reverse: CCCTGTGGATCTCACTGTCC. qPCR data were normalized to the mouse Top1 reference gene expression amplified using the following primers: Top1: Forward: GCCTCCATCACACTA-CAGCA and Reverse: TTCGCTGGTACATTCTCATCA. qPCR data were analyzed using the LightCycler 480 software release 1.5.0 and Excel program, as described previously.<sup>18</sup>

#### *Immunohistochemistry*

Histological analysis was performed on aortas (from aortic root to the bifurcation of the iliac arteries), pancreas, liver, and kidneys. The organs were dissected and fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 24 hours. Fixed tissues were dehydrated in graded ethanol and xylene and embedded in paraffin. Sequential sections of 4µm were mounted onto superfrost-plus glass slides. After de-paraffinization, sections were stained using standard procedures.<sup>24</sup> Sections were incubated in 0.1mg/ml pronase before incubation with CD31 (PECAM) and Lyve-1 antibodies. The monoclonal 1A4 antibody (alpha-SM-actin) and secondary antibody rabbit-anti-mouse peroxidase (RAM-PO) were first incubated to form 1A4-RAM-PO complex before adding it on sections following a previously described procedure.<sup>25</sup> The antibody complexes were visualized using 0.04% diaminobenzidine-tetra-hydrochloride (DAB). The following histochemical stainings were performed; Hematoxylin Eosin (HE) and Resorcin Fuchsin (RF) for elastin lamellae.

#### *Antibodies*

The following primary antibodies were used: CD31 (1:50, BD Bioscience, Erembodegem, Belgium), lyve-1 (1:1000, Reliatech, Wolfenbüttel, Germany),  $\alpha$ -Smooth muscle actin (1A4, 1:2000, Sigma-Aldrich, Zwijndrecht, Netherlands), anti-megalin (1:500, Pathology, Leiden, The Netherlands),<sup>26</sup> anti-Tamm Horsfall (Uromodulin) (1:3000, CAPEL, Durham, NC), anti-aquaporin-2 (1:300, Calbiochem, Amsterdam, The Netherlands). Secondary antibodies were: rabbit envision/ Horseradish Peroxidase (HRP) (undiluted; Dako, Heverlee, Belgium) and rabbit-anti-goat Ig/HRP (1:100, Dako, Heverlee, Belgium), goat anti-rat-biotin (1:200, BD Bioscience, Erembodegem, Belgium), goat serum (1:100, Brunschwig chemie, Amsterdam, The Netherlands), mouse-biotinylated (ABC-kit vectastain) (1:100, Brunschwig Chemie, Amsterdam, The Netherlands).

#### *X-Gal Staining*

Tissues of 3 days old *SM22Cre;ROSA26Cre* and *Tie2Cre;ROSA26Cre* mice and controls were stained whole mount for X-gal. Tissues were fixed for 2x 45 min in 0.2% glutaraldehyde, 1.5% paraformaldehyde, 2 mM MgCl<sub>2</sub>, 5 mM EGTA, 100 mM sodium phosphate pH 7.3. After fixation tissues were washed for 3x 20 min in wash solution (0.2% NP-40, 0.1% sodium deoxycholate, 2 mM MgCl<sub>2</sub>, 100 mM sodium phosphate pH 7.3). X-gal staining was performed for 48 hrs in 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 1 mg/ml X-gal (solved in dimethylformamide), 0.2% NP-40, 0.1% sodium deoxycholate, 2 mM MgCl<sub>2</sub>, 100 mM sodium phosphate pH 7.3. After washing for 3x 10 min in wash solution, tissues were post-fixed overnight in phosphate buffered 4% formaldehyde. Tissues were washed 2x 5 min in phosphate-buffered saline (PBS) and further processed for embedding by transferring them manually to 2x 60 min 50% ethanol, 2x 60 min 70% ethanol, 2x 60 min 95% ethanol, 2x 60 min 100% ethanol, 2x 60 min 100% 2-propanol. After warming to 60 °C the material was transferred to a pre-warmed mixture of 2-propanol/paraffin 1:1 for 2x 60 min and finally to paraffin for 3x 60 min. Sections of 4 µm were cut and counter stained with 0.1% Nuclear Fast Red and 5% Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> for 5-10 seconds.

### *Tail cuff measurements and osmotic pumps implantation*

Systolic blood pressure and heart rate were measured by the tail cuff method (BP2000, Visitech), at 37°C to dilate the tail artery, 5 times weekly and averaged for each week. *SM22-Pkd1<sup>del/del</sup>* and control wild type mice (50% *Pkd1<sup>lox/lox</sup>* and 50% *SM22Cre*), 30 weeks old, were habituated to the measurement for 3 weeks before implantation of the pumps and then monitored during about 4 weeks. The osmotic pumps (Alzet model 2004) were implanted subcutaneously on the back of the animals and continuously delivered a dose of 0.61 mg/kg/day of angiotensin II (Sigma) in PBS (Phosphate Buffer Saline). Systolic blood pressure and heart rate were recorded simultaneously. Significance of the 2 groups was tested with a permutation test (non parametric test) at a threshold of 5%; StatXact software).

To study the effects of elevated blood pressure on vessel structure, the thoracic aortas of control (*Pkd1<sup>lox/lox</sup>* and *SM22Cre*) and *SM22-Pkd1<sup>del/del</sup>* mice were observed. Fixation was done by perfusion with Bouin's fixative (formalin/picric acid) in the animal during 10 min and after removal from the animal in 10% neutral buffered formalin. Tissues were paraffin-embedded, sectioned, and stained with combined orcein (dark elastin staining) and picro-indigo-carmin (blue collagen staining).

### *Wire-myography*

Two-millimeter long segments of thoracic aorta were mounted on a wire-myograph (DMT, Aarhus, DK) (isometric myography).<sup>27</sup> Two steel wires (40 µm in diameter) were inserted into the artery lumen and fixed to a force transducer and a micrometer, respectively. A normalization procedure was used to evaluate the internal circumference that the aorta would have under a transmural pressure of 100 mmHg (IC100). To obtain a maximal and uniform response, all experiments were carried out at 90% of this internal circumference (IC90). Arteries were bathed in a physiological solution (PSS) composed of (in mM): NaCl 119; KCl 4.7; CaCl<sub>2</sub> 2.5; MgSO<sub>4</sub> 1.17; NaHCO<sub>3</sub> 25; KH<sub>2</sub>PO<sub>4</sub> 1.18; EDTA 0.027; glucose 5.5, oxygenated with a mixture of CO<sub>2</sub> (5%) and O<sub>2</sub> (95%), or the same PSS solution containing 80 mM KCl (NaCl substituted by KCl), named PSS 80K. Before each experiment, the contractility of the muscle was tested using the PSS 80K solution and then phenylephrin (1 µM) in standard PSS. The integrity of the endothelium was assessed by verifying that at least a 70% relaxation effect was obtained with acetylcholine (5 µM) after a precontraction by phenylephrin (1 µM). For each mouse, between 2 to 4 responding aorta rings were measured and averaged, error bars indicates the standard error to the mean, n indicates the number of mice. Significance was tested with a permutation test (R; R Development Core Team: <http://www.r-project.org/>) at a threshold of 5% (\*), 1% (\*\*), and 1‰ (\*\*\*). Cumulative dose response curves of phenylephrin, serotonin (5HT) and U46619 (thromboxan A2 agonist) were realized with extensive washout in between each curve. The cumulative dose response curve of acetylcholine was made after a precontraction with phenylephrin (1 µM). Experiments were performed on heterozygous *SM22-Pkd1<sup>del/+</sup>* mice and controls (*SM22Cre* and *Pkd1<sup>lox/+</sup>*), homozygous *SM22-Pkd1<sup>del/del</sup>* mice and their controls (*SM22Cre* and *Pkd1<sup>lox/lox</sup>*) at 11 weeks, as well as heterozygous *SM22-Pkd1<sup>del/+</sup>* mice and their controls and the germ-line *Pkd1<sup>del/+</sup>* and wild types, on mice aged 40 weeks.

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# **Chapter 6**

## **Summary and Discussion**

## Summary and Discussion

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most common inherited disorder of the kidneys that is characterized by the growth of numerous fluid-filled cysts in both kidneys. PKD cysts can enlarge the kidneys while replacing much of the normal structure, resulting in reduced kidney function and leading to kidney failure. Genes responsible for the disease are *PKD1* and *PKD2*. The ADPKD patients show widespread variations in disease severity ranging from rare cases showing massively enlarged cystic kidneys in utero, through more typical presentation with end stage renal disease (ESRD) in the fifth or sixth decade, to cases with sufficient kidney function until old age. Several reasons are suggested for this variation, like the location of the mutation in the *PKD1* and/or *PKD2* gene, mutations in other modifying genes, and environmental factors.<sup>1,2</sup>

The molecular and cellular mechanisms responsible for cyst formation are not known, but there are a lot of hypotheses. Cystogenesis is characterized by epithelial cell proliferation, excessive secretion of solute and fluid into the cyst lumen and accumulation of matrix proteins in the renal interstitium, resulting into loss of functional renal tissue and interstitial fibrosis.

In this thesis we studied these mechanisms using predominantly a hypomorphic *Pkd1* mouse model (C57Bl6/J-*Pkd1*<sup>nl/nl</sup>), which is an orthologous model for polycystic kidney disease, with a rapidly progressive phenotype (chapter 2).<sup>3</sup> It shows an early mortality which is either caused by rupture of dissecting aneurysms, which are found in large vessels like the aorta and mesenteric artery at the age of 3 to 4 weeks (chapter 4), or by renal failure as blood urea levels were moderately elevated at the same age.

While the C57Bl6/J-*Pkd1*<sup>nl/nl</sup> live for a few weeks, the *Pkd1*<sup>nl/nl</sup> mice backcrossed on 129/Ola background live longer and F1 mice of a homogeneous C57Bl6/J;129/Ola background have been analyzed up to one year. The C57Bl6/J;129/Ola -*Pkd1*<sup>nl/nl</sup> model shows the same progressive renal phenotype at 4 weeks as the C57Bl6/J-*Pkd1*<sup>nl/nl</sup> however, the former model shows a peak of the cystic index and kidney/body weight at the age of 4 to 5 weeks which then declines during a few weeks and increases again (H. Happé, personal communications). An explanation for the decreased cystic index might be that the cysts fold in at this age, thereby reducing the number of cysts. At these regions fibrotic tissue is formed. Indeed transforming growth factor beta (TGFβ target genes, which are involved in fibrosis, also shows a peak at almost the same time point.

The genetic background might also influence the development of aneurysms in these mice. *Pkd1*<sup>nl/nl</sup> mice, which had a 129/Ola origin, when backcrossed on C57Bl6 background for 3 to 6 times show dissecting aneurysms in almost every mouse. However, further crossing on C57Bl6 or backcrossing on the 129/Ola background probably vanish or reduce the prevalence of this phenotype. The fact that the *Pkd1*<sup>nl/nl</sup> gene originates from 129/Ola background, suggests also an epistatic effect of the flanking gene region, which become smaller upon backcrossing on C57Bl6. Indeed, ADPKD patients, even in the same family, do not all develop vessel abnormalities indicating that inactivation of the *Pkd1* gene is not sufficient to initiate aneurysm formation and a certain combination of modifying genes must be present.

All mouse models homozygous for a *Pkd1* deletion die around E15 presenting severe cystic kidneys, indicating that polycystin1(PC1) is required for normal kidney development.<sup>4-7</sup> The C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice, however, express low levels of *Pkd1*, are viable, and exhibit grossly normal kidney structure but with dilated proximal tubules at birth (chapter 2). The first cysts develop from the fully mature proximal tubules around birth. This is followed by a phase of distal tubule and collecting duct cyst formation and progressive enlargement leading to renal failure. The same pattern might also occur in ADPKD patients. Prenatal ultrasound examination revealed hyperechogenic cortex that occurs in the third trimester. This hyperechogenic cortex is probably related to the presence of multiple microcysts within the renal cortex, which are most likely proximal tubules as the majority of the cortical tubules are proximal tubules.<sup>8</sup> Indeed, one study reported proximal cysts in a hypomorphic *PKD1* fetus.<sup>9</sup>

This pattern of nephron segment-specific cyst formation during nephrogenesis could be a reflection of *Pkd1* expression. In immature proximal tubules, *Pkd1* expression is high and declines progressively in mature proximal tubules but starts to increase in the loop of Henle, distal tubules, and collecting ducts, a pattern that persists in adult kidneys. This pattern is also found in other models of ADPKD or ARPKD and in humans.<sup>10-20</sup>

One of the most interesting features of ADPKD is cellular growth. Due to an unknown mechanism, a subset of epithelial cells escape the normal regulatory mechanism and start to grow and expand resulting in cyst formation. A variety of studies indicate increased cell proliferation in the cystic kidneys of animal models and ADPKD patients. In the hypomorphic model, the C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice, the overall proliferation index in the renal epithelium at neonatal stages was comparable to wild type mice. Proliferation is much higher in the kidneys of 7- and 13 day-old mice compared to older mice, a phenomenon related to a developmental switch in kidney maturation.<sup>21</sup> At this age proliferation is highest in the cortico-medullary region, which is a major growth zone for medullary structures and the region from which the majority of cysts are derived in C57Bl6/J-*Pkd1*<sup>nl/nl</sup>. Furthermore, conditional knockout models of *Pkd1* have identified that inactivation of both alleles before 13 post-natal days results in severe cystic disease, whereas inactivation after 14 post-natal days causes much milder disease.<sup>21,22</sup> Beside the developmental switch, renal injury seems to increase proliferation and increase cystic index of conditional knockout *Pkd1* models. This phenomenon coincides with aberrant planar cell polarity and increased Hippo signaling, both of which are also involved in tumorigenesis.<sup>23,24</sup>

Beside proliferation, another driving force for cyst enlargement is transepithelial fluid and Cl<sup>-</sup> secretion mediated by NKCC1 and CFTR.<sup>25</sup> In cystic epithelial cells chloride enters across basolateral NKCC1 cotransporters, driven by the sodium gradient generated by basolateral Na-K-ATPase, and exits across apical protein kinase A (PKA)-stimulated cystic fibrosis transmembrane conductance regulator (CFTR). Accumulation of chloride within the cyst lumen drives sodium and water secretion down transepithelial potential and osmotic gradients. In C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice, the expression pattern of NKCC1 in cystic kidneys is similar to the pattern observed in wild type kidneys with a relatively strong expression in proximal tubule-derived cysts, but only weak expression in cysts derived from distal tubules and collecting ducts (chapter 2). The fact that in this mouse

model, the first cysts are derived from the proximal tubules, suggests that transepithelial fluid and Cl<sup>-</sup> secretion is important in cyst formation at younger ages.

In addition to cysts formation, excessive fibrotic tissue is responsible for the loss of normal renal tissue. We show that TGFβ signaling, the major fibrogenic cytokine, is increased in C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice during cyst expansion (chapter 3).<sup>26</sup> Cytoplasmic TGFβ expression and nuclear localization of P-Smad2 were detected in cystic epithelial cells as well as in interstitial cells suggesting that both cell types synthesize and respond to TGFβ. This was accompanied by increased matrix synthesis as well as inadequate matrix degradation detected by increased synthesis of collagen, fibronectin, PAI-1 and MMP's, suggesting a complex regulation of matrix remodeling with an imbalance towards accumulation of matrix proteins. Targeting TGFβ by knocking out *Alk-5*, a gene encoding a TGFβ receptor, in a *Pkd1* conditional knock-out mice did not significantly prevent the expressing of the TGFβ target genes (W. Leonhard, personal communication). This suggests that this pathway can still be activated via an alternative way; therefore inhibiting more than one target in this pathway is essential to retard or inhibit fibrosis.

Apart from the cyst formation in the kidneys, the *Pkd1*<sup>nl/nl</sup> mice, depending on genetic background, provide also a model to study aortic dissecting aneurysm formation in ADPKD, confirming the role of *Pkd1* in dissecting aneurysm formation (chapter 4).<sup>27</sup> Our mouse model represents a very severe phenotype of ADPKD with dissections in the aorta. We hypothesize that upon reducing the expression of PC1, the stability of the contact between smooth muscle cells (SMC) and extracellular matrix molecules is reduced. This may disturb some cellular events in the SMCs and endothelial cells (EC) resulting in cell proliferation and increased extracellular matrix synthesis, which accumulate between the elastin lamellae and weaken the vessel wall. Finally, the vessel wall tears leading to dissection. However, intact regions of the aorta were still present and the overall construction of the aorta with regard to the elastin lamellae was normal. This indicates that PC1 is probably not implicated in vessel wall development but rather maintains the structural integrity of the vasculature.

We further analyzed the consequences of the specific deletion of *Pkd1* in SMCs (*SM22-Pkd1*<sup>del/del</sup>), which are the major component of the media (chapter 5).<sup>28</sup> These mice, however, did not show any structural alterations of the aortic vessel wall. Moreover, disruption of *Pkd1* simultaneously in SMCs and ECs (*SM22;Tie2-Pkd1*<sup>del/del</sup>) did not result in structural blood vessel alterations, even though the recombination levels were high in blood vessels as shown by the *Cre* reporter and by strongly reduced *Pkd1* mRNA levels. These data suggest that additional triggers are needed to induce or to accelerate the phenotype. We can think of several explanations why the C57Bl6/J-*Pkd1*<sup>nl/nl</sup> mice and *Pkd1* knock-out mice, show structural abnormalities of the blood vessels, while the *SM22;Tie2-Pkd1*<sup>del/del</sup> mice do not, like the influence of the different genetic background of mouse models used and contribution of other cell types than SMCs and ECs in which *Pkd1* is not disrupted in the *SM22;Tie2-Pkd1*<sup>del/del</sup> mice.

Although we did not observe spontaneous structural alterations in the aorta's of *SM22-Pkd1*<sup>del/del</sup> mice, these mice showed a decreased myogenic tone in resistance arteries. Interestingly, the ratio of PC1/PC2, which is disturbed in the mutants, is of crucial

importance for the regulation of stretch-activated ion channels (SACs) in arterial myocytes. Activation of SACs regulates myocyte contraction and thereby the vascular tone.<sup>29</sup> When we implanted angiotensin II releasing pumps to induce hypertension, the heart rate significantly slowed down in all genotypes. This decrease in heart rate is the result of adaptation of the cardiac outflow to increased peripheral resistance and/or to the central nervous system effect of angiotensin. Interestingly, AngII-induced decrease in heart rate was significantly less pronounced in *SM22-Pkd1<sup>del/del</sup>*. According to the classical Guyton model, without autoregulation (i.e. myogenic tone), cardiac output in response to hypertension is predicted to remain high.<sup>30</sup> This model fits nicely with our data as anticipated by a loss of myogenic tone in *SM22-Pkd1<sup>del/del</sup>* mice.<sup>29</sup>

The *SM22-Pkd1<sup>del/del</sup>* provides a model to study other extra-renal manifestations of ADPKD as we observed cyst formation in pancreas, liver, and kidneys. Expression of LacZ matched with the sites of cyst formation and was found in pancreatic ducts and hepatic bile ducts, which are the origin of the cysts. In the kidneys, only glomerular cysts were found and recombination did occur in the parietal layer of Bowman's capsule of glomeruli. Glomerular cysts can also be found as part of ADPKD, predominantly in fetal kidneys but also in adult end-stage kidneys in addition to tubular cysts. In *Pkd1* mutant mouse models glomerular cysts were only observed in *Pkd1* knock-out mice but not in hypomorphic mice that have low levels of *Pkd1* gene expression, suggesting that total absence of *Pkd1* is necessary to induce the formation of glomerular cysts. How cysts arise in *SM22-Pkd1<sup>del/del</sup>* is not entirely clear. Defects in cilia signaling, cellular signaling, cell-cell and cell-matrix interactions, which have been observed in ADPKD tubular cysts, probably play an important role. In addition, as PC1 is known to affect migration and proliferation, the absence of *Pkd1* might disturb the recruitment of podocytes from the glomerular parietal epithelial cells, which proliferate and migrate into the glomerular tuft and differentiate into podocytes.<sup>31</sup>

Significant progress has been made over the last few years towards a better understanding of the pathogenesis of ADPKD. Our mouse models and data contribute by describing and analyzing cyst and aneurysm formation in mouse models of PKD. Polycystins are suggested to be involved in a variety of cellular functions. This is confirmed by the different processes which are deregulated upon mutation in a *PKD* gene, like proliferation, ion channel expression, extracellular matrix production and cilia mechano-sensing. Therefore, a multi-target targeted approach is crucial to slow down cyst formation. Indeed, curcumin, which has a beneficial effect on a wide variety of inter and intracellular signaling pathways, many of which are involved in cysts formation, seems to retard renal failure.<sup>32</sup>

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## Nederlandse Samenvatting

Autosomaal dominant polycysteus nier ziekte (ADPKD) is een veel voorkomende erfelijke nierziekte met een prevalentie van 1:400-1:1000. Patiënten hebben vele blaasjes gevuld met vocht (Cysten) in de nieren. De afmetingen hiervan kunnen variëren van minder dan 1 mm tot 1 cm. Naarmate deze blaasjes groeien, zullen zij het functioneren van de nieren bemoeilijken en samen met verbindweefseling uiteindelijk leiden tot chronisch nierfalen. De meeste patiënten bereiken het eind stadium van nierfalen in het 5de of 6de decennium en hebben uiteindelijk nierdialyse nodig. Daarnaast, kunnen deze patiënten ook lijden aan andere complicaties, zoals cysten in de lever, aneurysmata in de arteriële bloedvaten (aandoening waarbij de wand van de bloedvaten zwak wordt en kan scheuren) en hoge bloeddruk. De oorzaak van cystenieren is een defect van de genen *PKD1* of *PKD2*. Deze genen regelen de aanmaak van de eiwitten polycystine-1 en polycystine-2 (PC1 en PC2). Deze eiwitten spelen een rol onder andere bij cel-cel en cel-matrix contacten en signaaloverdracht waardoor ze opbouw van de het weefsel onderhouden. Wanneer de *PKD* genen niet goed zijn, dan zal er minder of zullen er afwijkende eiwitten aanwezig zijn. Daardoor kunnen de eiwitten hun functie niet meer voldoende uitvoeren en worden de cellen in de nierbuisjes ontregeld, 1) de cellen delen nu vaker, zodat er meer cellen ontstaan 2), sommige zouten en vloeistoffen worden de verkeerde richting opgepompt zodat de cysten groter worden, 3) en er zetten zich meer eiwitten aan de onderkant van deze cellen af, waardoor er meer bindweefsel ontstaat. Deze processen zorgen er voor dat de smalle buisjes van de nefron verwijden en er ontstaan cysten.

Een aantal aspecten van de ziekte hebben we in verschillende muismodellen bestudeerd, namelijk in muizen waarin we het *Pkd1* gen specifiek in niercellen hebben uitgeschakeld, en muizen waarin in alle lichaamscellen een te lage activiteit van dit gen is.

In hoofdstuk 2 is de cysten ontwikkeling bestudeerd in het *Pkd1* muis model met een zeer lage genactiviteit in alle cellen van alle organen. De celdeling, celdood, aanmaak van eiwitten tussen de cellen en ionen kanalen die de vochtophoping in de cysten stimuleren zijn bestudeerd. Dit muis model toont een snelle progressieve ziekte verloop met weinig bindweefsel. Celdeling en celdood zijn niet verhoogd vergeleken met de gezonde muizen.

In hoofdstuk 3 wordt de relatie van TGF-, een belangrijk eiwit dat de aanmaak van bindweefsel reguleert, bestudeerd in verschillende muismodellen met PKD. TGFβ aanmaak en activatie van de signaaltransductie route is verhoogd in de milde (weinig cysten, maar soms grote cysten) en in progressieve fase (een groot gedeelte van de nier bestaat uit cysten) van cystenieren. In het begin fase, waarbij de meeste cysten klein zijn, is dit eiwit niet erg actief.

Behalve nierfalen lijdt 1 tot 10% van de ADPKD patiënten ook aan aneurysmata. Dit is een aandoening van de vaten waarbij de vaatwand zwak wordt en kan scheuren. Patiënten overlijden dan aan interne bloedingen. Het verlagen van de PC1 in de hoofdslagader (aorta) ontregelt de cellen in de vaatwand waardoor dit zwak wordt en scheurt. In hoofdstuk 4 wordt deze aandoening in hetzelfde muismodel als in hoofdstuk 2, bestudeerd. Deze studie toont een directe relatie tussen de *Pkd1* en vaatwand behoud.

In de vaatwand zijn er voornamelijk twee soorten cellen, endotheel cellen voor de binnenbekleding van de vaatwand en gladde spiercellen binnen in de vaatwand. In hoofdstuk 5, is PC1 alleen in de gladde spieren verlaagd. Heel opmerkelijk, leidt dit niet tot veranderingen in de vaatwand maar ontstaan er cysten in de alvleesklier, lever en nieren. Wanneer *Pkd1* werd verlaagd in de endotheel cellen en in gladde spier cellen samen, leidde dat ook niet in duidelijk afwijkingen in de vaatwandstructuur. Als volgende stap werd in deze muizen de bloeddruk verhoogd door medicatie (Angiotensine II). In de gezonde situatie wordt de hartslag verlaagd als reactie op de hogere bloeddruk, maar in deze mutant muizen werd de hartslag minder verlaagd dan in de gezonde situatie. Dit toont aan dat aanpassingen van de hartslag aan de bloeddruk is verstoord in deze muizen.

## **Curriculum Vitae**

De auteur van dit proefschrift, Sabine Hassane, is geboren op 3 oktober 1978 te Koeweit. Van 1993 tot 1998 heeft zij VWO gevolgd aan het Erasmus College te Zoetermeer, en van 1998 tot 2002 heeft zij de studie Biomedische Wetenschappen aan de Universiteit Leiden gevolgd. Tijdens de studie heeft zij 2 wetenschappelijke stages gevolgd aan de Leidse Universitaire Medisch Centrum:

- 1- Het effect van PPAR $\gamma$  agonisten op de tumorprogressie en de angiogenese in vitro en in vivo. Afdeling Endocrinologie.
- 2- HPV16 E2 and E6 specific memory Th immunity in healthy individuals. Afdeling Hematologie.

In 2003 begon Sabine Hassane als promovendus op de afdeling Humane Genetica van het Leidse Universitaire Medisch Centrum om onderzoek te doen aan kyste nieren en aneurysmata in autosomaal dominant polycysteus nier ziekte.

In 2009 heeft zij een carrière switch gemaakt naar het onderwijs en volgt zij momenteel een eerstegraads docent opleiding biologie aan de universiteit Leiden.

## List of publications

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## Nawoord

Het voltooiën van dit boekje is voor mij een bijzonder moment. Ik zou graag iedereen voor hun bijdrage aan de totstandkoming van dit boekje willen bedanken.

Mijn dank gaat in het bijzonder uit naar mijn collegas op de afdeling humane genetica: Nanna, Funda, Paola, Wouter, Hester, Irma en Hans. Verder was dit onderzoek niet mogelijk geweest zonder de goede samenwerking met de afdelingen anatomie (Marco en Conny), pathologie (Emile, Annemieke en Hans) en Gaubius Laboratorium TNO (Roeland en Natascha).

Mijn moeder en vader, mijn zus Imaan en mijn broers Adham, Walid, Hassan en Ahmed, veel dank voor jullie steun, zonder jullie zou ik dit niet kunnen doen.

*Alhamdulillah rab al alamien.*

Sabrine Hassane



