



Molecular mechanisms underlying vascular problems in Autosomal Dominant Polycystic Kidney Disease

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RESUMO

A Doença Poliquística Renal Autossômica Dominante (ADPKD do inglês *Autosomal Dominant Polycystic Kidney Disease*) é uma doença crônica renal hereditária que afeta 1 em 400-1000 recém-nascidos em todo o mundo. É causada por mutações, maioritariamente, nos genes *PKD1* (78% dos casos) e *PKD2* (15% dos casos) que codificam as proteínas policistina-1 (PC1) e policistina-2 (PC2). Estas encontram-se localizadas em diversas estruturas celulares, porém, especialmente, a PC1 e a PC2 formam um complexo mecanossensorial na membrana dos cílios primários no epitélio renal, atuando respetivamente como um mecanossensor de estímulos extracelulares (como o fluxo de urina) e como um canal não seletivo de Ca^{2+} . Este complexo é essencial para a homeostase do Ca^{2+} e, consequentemente, para a diferenciação e manutenção do epitélio renal. Adicionalmente, recentemente, também foram descobertos dois novos genes ligados a casos de ADPKD: *GANAB* e *DNAJB11* (3% e 1,2% dos casos, respetivamente), cujas proteínas se pensa estarem associadas à maturação e deslocação de proteínas transmembranares, particularmente a PC1.

Na ADPKD, a disrupção destas proteínas leva a uma diminuição nos níveis basais de Ca^{2+} intracelular, provocando um aumento dos níveis de AMP cíclico (cAMP) e a desregulação de várias outras vias de sinalização, iniciando a cystogénese. Desta forma, desenvolvem-se múltiplos quistos renais cheios de fluído, cujo epitélio apresenta várias características patológicas, incluindo proliferação aumentada, apoptose aumentada, remodelação da matriz extracelular, fenótipo secretor, metabolismo desregulado e polaridade celular aberrante. A formação e inflação contínuas destes quistos, que constitui a principal manifestação clínica desta doença, destrói gradualmente o parênquima renal e, eventualmente, leva à insuficiência renal. A severidade da doença varia consoante o gene afetado, sendo que mutações no gene *PKD1* estão associadas um fenótipo mais grave.

No entanto, as manifestações da ADPKD não se limitam a este órgão; na verdade, é uma doença sistémica que leva a muitas complicações extrarrenais, como doença poliquística hepática, desenvolvimento de quistos no pâncreas, membrana aracnoide e vesícula seminal, diverticulose e diverticulite do cólon. Mas para além disto, esta doença também está associada a anomalias vasculares, que podem levar a aneurismas intracranianos, disseção e regurgitação da aorta, valvulopatias e hipertensão, que foram o foco deste projeto. Evidências na literatura mostram que a PC2 é expressa nas células endoteliais e musculares lisas vasculares, sugerindo uma importante função desta proteína no sistema cardiovascular. De facto, foi demonstrado que, nas células endoteliais vasculares, a ativação da PC2, pela tensão na parede dos vasos, resulta na dilatação do músculo liso e relaxamento vascular induzido pelo fluxo. Além disso, a PC2 também tem sido implicada na deteção da pressão de volume nas células do músculo liso vascular e na resposta miogénica. No entanto, a contribuição do canal PC2 para as alterações vasculares observadas em pacientes com ADPKD permanece incerta.

Tendo em conta que a sequência e função desta policistina no peixe-zebra (*Danio rerio*) é altamente conservada com o ortólogo humano e dado o potencial deste organismo modelo para estudar a formação e doença da vasculatura, o nosso grupo postulou que a ausência de *pkd2* levaria a anomalias vasculares nestes animais. Desta forma, escolhemos usar linhas de peixe-zebra para realizar as nossas observações *in vivo* da vasculatura embrionária, através de microscopia estereoscópica de fluorescência. Assim sendo, caracterizamos a vasculatura normal destes animais e as anomalias cardiovasculares causadas pela falta de *Pkd2*, usando algumas ferramentas. Primeiramente, usando um morfolino bloqueador de tradução do mRNA alvo, injetado em embriões (no estágio de uma célula) da linha *Tg(fli1:eGFP; gata1a:DsRed)^{y1;sd2}*. Esta linha transgénica expressa GFP sob o controlo do promotor do gene *fli1*, assim como, dsRed sob o controlo do promotor do gene *gata1a*, e, portanto, marca células

endoteliais vasculares e glóbulos vermelhos ao longo da embriogénese, respetivamente. O que nos permitiu discriminar anomalias na vasculatura relacionadas com a falta de Pkd2 até às 48 horas após a fertilização (hpf). E, por outro lado, gerando a linha mutante *curly up* (*cup*)^{tc321} × *Tg(fli1:eGFP; gata1a:DsRed)*^{y1;sd2}, cruzando indivíduos heterozigóticos da linha mutante *curly up* (*cup*)^{tc321} com indivíduos da linha *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2}. Os primeiros possuem uma mutação em *cup*, que codifica o ortólogo do peixe-zebra do gene *PKD2* humano, levando a uma proteína truncada não-funcional. Esta linha permitiu-nos observar os embriões às 48, 72 e 96 hpf e, mais uma vez, com a marcação das células endoteliais vasculares e glóbulos vermelhos, para analisar anomalias vasculares.

Os nossos resultados evidenciaram que os *pkd2-morphants* apresentavam várias anomalias cardiovasculares, o que foi consistente com o que era esperado e o que fora reportado em alguns trabalhos de investigação, que incluíam: defeitos *in situ* cardíacos, edema cardíaco, hemorragias intracranianas e hidrocefalia; que podem estar ligadas à interrupção das vias dependentes de *pkd2* nos estágios iniciais de desenvolvimento, nomeadamente de vias responsáveis pela manutenção e função da vasculatura, que envolvem a matriz extracelular, a hipertensão e inflamação. Os defeitos observados no plexo da veia caudal e nos vasos intersegmentares não foram significativamente associados à falta de Pkd2, sugerindo um efeito *off-target* do morfolino ou possivelmente um ligeiro atraso no desenvolvimento associado ao uso de morfolino. No entanto, nem todos os fenótipos foram encontrados em *cup*^{-/-} mutants, mesmo aquando da sua observação em estádios de desenvolvimento posteriores. De facto, o edema cardíaco foi o principal defeito cardiovascular detetado nestes animais. As razões por trás da ausência de alguns defeitos vasculares em *cup*^{-/-} mutants que foram observados em *pkd2-morphants*, podem ser atribuídas a efeitos *off-target* do *pkd2*-augMO em *pkd2-morphants* ou à significativa contribuição materna de mRNA de *pkd2* presente em *cup*^{-/-} mutants já descrita na literatura, que pode atenuar fenótipos defeituosos. De facto, esta pode ser a principal contribuinte para as nossas observações em *cup*^{-/-} mutants, visto que, à medida que a contribuição materna de Pkd2 foi progressivamente silenciada, o edema cardíaco foi agravando ao longo do desenvolvimento embrionário. Isto demonstrou a necessidade de uma investigação mais aprofundada dos mecanismos moleculares pelos quais o *pkd2* poderá influenciar a vasculatura. De facto, para entender melhor os nossos resultados, será importante realizar no futuro ensaios de imunolocalização em vasos sanguíneos de *pkd2-morphants* e *cup*^{-/-} mutants, e respetivos controlos assim como continuar a observar *cup*^{-/-} mutants até aos 6 dias após a fertilização, momento em que morrem devido a complicações de malformação.

Adicionalmente, foram realizadas medições do diâmetro da aorta dorsal em 5 diferentes localizações: 5°, 10°, 15°, 20° e 25° miótomo (unidade muscular do peixe-zebra). As medições do diâmetro da aorta dorsal de 48 hpf em ambos *pkd2-morphants* e *cup*^{-/-} mutants mostraram que este vaso apresentava um calibre significativamente reduzido, o que parece ser causado por vasoconstricção. Esta diferença não foi verificada em estádios de desenvolvimento posteriores em *cup*^{-/-} mutants, o que sugere que a Pkd2 poderá ter um papel mais determinante na vasculatura em estádios iniciais da embriogénese ou que as forças mecânicas que regulam a remodelação vascular após as 48 hpf são suficientes para esta ocorrer e que estes mecanismos podem ser independentes da função da Pkd2. Para além disto, os nossos resultados preliminares mostram claramente que a análise por microscopia confocal pode oferecer uma caracterização mais precisa destas anomalias.

Concluindo, o nosso trabalho demonstra que a linha de peixe-zebra que criámos: *curly up* (*cup*)^{tc321} × *Tg(fli1:eGFP; gata1a:DsRed)*^{y1;sd2} é um ótimo modelo não só para observar e caracterizar problemas vasculares em vários estádios do desenvolvimento embrionário, mas também para estudar os mecanismos moleculares subjacentes a essas alterações devido à perda de *pkd2*.

Palavras-chave: Doença Poliquística Renal Autossômica Dominante (ADPKD); Policistina-2 (PC2); peixe-zebra (*Danio rerio*); problemas vasculares; hipertensão

ABSTRACT

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is an inherited chronic kidney disease that is estimated to affect 1 in 400-1000 newborn worldwide. It is caused by mutations mainly in *PKD1* (78%) and *PKD2* (15%). Recently, two additional genes were found to be linked to cases of ADPKD: *GANAB* and *DNAJB11*. The disruption of encoded proteins causes cytotgenesis resulting in several fluid-filled kidney cysts, which is the main clinical manifestation of this disease. The continuous formation and inflation of these cysts, gradually destroy renal parenchyma and eventually leads to renal failure. However, ADPKD is not limited to this organ; indeed, it is a systemic disease that leads to many extrarenal complications. This project was focused on vascular abnormalities, which can lead to intracranial aneurysms, aorta dissection and regurgitation and hypertension.

Evidence from literature has shown that Polycystin-2 (PC2) is expressed in the endothelial and smooth muscle cells, suggesting an important function of this protein in the cardiovascular system. Still, the contribution of the PC2 channel to vascular changes observed in ADPKD remains unclear. Knowing that this polycystin sequence and function in zebrafish (*Danio rerio*) is highly conserved with human ortholog and the potential of this model to study vasculature formation and disease, we postulated that *pkd2* loss would lead to vascular anomalies in these animals.

We chose to use *Tg(fli1:eGFP; gata1a:DsRed)^{y1;sd2}* zebrafish line to perform our *in vivo* embryo vasculature observations. On the other hand, we studied the impact of the loss of *pkd2* expression by using a *pkd2* translational blocker morpholino and by generating the *curly up (cup)^{tc321} × Tg(fli1:eGFP; gata1a:DsRed)^{y1;sd2}* mutant line. Using these tools, we characterized the normal vasculature of these animals and the cardiovascular anomalies caused by the lack of Pkd2, by fluorescence stereomicroscopy. Our results showed that *pkd2*-morphants presented several cardiovascular anomalies: heart situs defects, cardiac edema, hemorrhages and hydrocephalus, which may be linked to the disruption of *pkd2*-dependent pathways in the early stages of development. However, not all phenotypes were found in *cup^{-/-}* mutants, even when observing further developmental stages. Indeed, cardiac edema was the main cardiovascular defect detected in these animals. This supports the significant maternal contribution of *pkd2* mRNA that has been described in the literature, which may attenuate defective phenotypes. We think that this may be the main contributor to our observations in *cup^{-/-}* mutants, given that as the Pkd2 maternal contribution was progressively silenced, cardiac edema was aggravating throughout embryonic development. This shows the need for further investigation of molecular mechanisms by which *pkd2* could influence vasculature. Indeed, to better understand our findings, it will be important to perform in the future immunolocalization assays in zebrafish blood vessels of both *pkd2*-morphants and *cup^{-/-}* mutants, and respective controls. Additionally, 48 hpf dorsal aorta diameter measurements in both *pkd2*-morphants and *cup^{-/-}* mutants showed that this vessel had a significant reduced caliber, which seems to be caused by vasoconstriction. Such difference was not verified at later developmental stages in *cup^{-/-}* mutants which suggested either that Pkd2 had a more determinant role in the vasculature at earlier stages of embryogenesis or that the mechanical cues that regulate vascular remodeling after 48 hpf are sufficient for it to occur and may be independent of Pkd2 function. Our preliminary results clearly show that confocal microscopy analysis may offer us a more accurate characterization of these anomalies.

Altogether, our work demonstrates that the zebrafish line we generated *curly up (cup)^{tc321} × Tg(fli1:eGFP; gata1a:DsRed)^{y1;sd2}* is a great model to not only observe and characterize vascular problems at several stages of embryonic development, but also to study the molecular mechanisms underlying these alterations due to *pkd2* loss.

Keywords: Autosomal Dominant Polycystic Kidney Disease (ADPKD); Polycystin-2 (PC2); zebrafish (*Danio rerio*), vascular problems; hypertension

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LIST OF ABBREVIATIONS

aa	amino acids
AA	Aortic Arches
AC	Adenylate Cyclase
ACE	Angiotensin-Converting Enzyme
ACeV	Anterior Cerebral Vein
acta2/ α sma	α -smooth muscle actin
ADKPD	Autosomal Dominant Polycystic Kidney Disease
AQP2	Aquaporin 2
ATP	Adenosine Triphosphate
BA	Basilar Artery
BCA	Basal Communicating Artery
BiP	Binding immunoglobulin Protein
B-Raf	Serine/threonine-protein kinase B-raf
CA	Caudal Artery
CaM	Calmodulin
cAMP	cyclic Adenosine Monophosphate
CCV	Common Cardinal Vein
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
<i>COL11A1</i>	Collagen Type XI Alpha 1 Chain human gene
<i>col11a1a</i>	Collagen type XI alpha 1a zebrafish gene
CrDI	Cranial Division of the Internal Carotid artery
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9
CT	Computed Tomography
CV	Caudal Vein
cvp	caudal vein plexus
DA	Dorsal Aorta
DCV	Dorsal Ciliary Vein
DLAV	Dorsal Longitudinal Anastomic Vessel

DMJ	Dorsal Middle Junction
DNA	Deoxyribonucleic acid
<i>DNAJB11</i>	DNAJB11 gene
DNAJB11	DNAJB11 protein
dpf	days post-fertilization
dsRed	Red fluorescent protein
e	ear
ECM	Extracellular Matrix
ENaC	Epithelial Sodium Channel
eNOS	endothelial Nitric Oxide Synthase
ER	Endoplasmic Reticulum
ERK5	Extracellular-signal-Regulated Kinase 5
ESRD	End-Stage Renal Disease
f	fin bud
<i>fli1</i>	fli1 proto-oncogene, ETS transcription factor or friend leukemia integration 1 transcription factor zebrafish gene
<i>GANAB</i>	Catalytic α -subunit of glucosidase II gene
GANAB	Catalytic α -subunit of glucosidase II protein
<i>gata1a</i>	gata binding protein 1a zebrafish gene
GFP	Green Fluorescent Protein
GFR	Glomerular Filtration Rate
GII α	Catalytic α -Subunit of Glucosidase II protein
GII β	Regulatory β -Subunit of Glucosidase II protein
GPS	G Protein-coupled Receptor Proteolytic Site
h	heart
HEK293	Human Embryonic Kidney 293 cells
<i>HNF-1β</i>	HNF1 Homeobox B gene
HPD	Histidyl-Propyl-Aspartic acid
hpf	hours post-fertilization
HPV	Hepatic Portal Vein
ICA	Intracranial Aneurysm

IP3	Inositol-1,4,5-triphosphate
IP3R	Inositol-1,4,5-triphosphate Receptor
j	forming jaw
JG	Juxtaglomerular
Klf2a	Kruppel-like factor 2a
KV	Kupffer's Vesicle
LDA	Lateral Dorsal Aorta
LDL-A	Low Density Lipoprotein-A
LRR	Leucine-Rich Repeat
MAPK/ERK	Mitogen-Activated Protein Kinase/Extracellular Regulated Kinase
MCeV	Middle Cerebral Vein
MDCK	Madin-Darby Canine Kidney cells
MOs	Morpholino Oligonucleotides
mRNA	messenger RNA
MS-222	Tricaine mesylate
MsV	Mesencephalic Vein
MtA	Metencephalic Artery
mTOR	mammalian Target of Rapamycin
MVP	Mitral Valve Prolapse
NGS	Next-Generation Sequencing
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
Nos2b	Nitric oxide synthase 2b
NV	Nasal Vein
PA	Pectoral Artery
PC1	Polycystin-1
PC2	Polycystin-2
PCP	Planar Cell Polarity
PCR	Polymerase Chain Reaction
PCS	Posterior Communicating Segment
PFA	Paraformaldehyde

PHBC	Primoridal Hindbrain Channel
PKA	Protein Kinase A
PKC	Protein Kinase C
PKD	Polycystic Kidney Disease
<i>PKD1</i>	Polycystin-1 human gene
<i>Pkd1</i>	Polycystic kidney disease 1 mouse gene
Pkd1	Polycystic kidney disease 1 zebrafish and mouse protein
<i>pkd1</i>	Polycystic kidney disease 1 zebrafish gene
<i>pkd1a/b</i>	Polycystic kidney disease 1 a and b zebrafish genes
<i>pkd1b</i>	Polycystic kidney disease 1 b zebrafish gene
<i>pkd1l1</i>	Polycystic kidney disease 1 like 1 zebrafish gene
<i>pkd1l2a</i>	Polycystic kidney disease 1 like 2 a zebrafish gene
<i>pkd1l2b</i>	Polycystic kidney disease 1 like 2 b zebrafish gene
<i>PKD2</i>	Polycystin-2 human gene
<i>Pkd2</i>	Polycystic kidney disease 2 mouse gene
Pkd2	Polycystic kidney disease 2 zebrafish and mouse protein
<i>pkd2</i>	Polycystic kidney disease 2 zebrafish gene
<i>pkd2l1</i>	Polycystic kidney disease 2 like 1 zebrafish gene
PLAT	Polycystin-1, Lipoxygenase, Alpha-Toxin
PLD	Polycystic Liver Disease
PMBC	Primordial Midbrain Channel
PrA	Prosencephalic Artery
<i>PRKCSH</i>	Protein Kinase C Substrate 80 K-H gene
PTU	1-Phenyl-2-Thiourea
PVC	Posterior Cardinal Vein
RAAS	Renin-Angiotensin Aldosterone System
RAS	Renin-Angiotensin System in zebrafish
REJ	Receptor for Egg Jelly
RER	Rough Endoplasmic Reticulum
RNA	Ribonucleic Acid
RT-PCR	Real-Time Polymerase Chain Reaction

RyR	Ryanodine Receptor
SAH	Subarachnoid Hemorrhage
Se	Intersegmental vessels
<i>SEC63</i>	SEC63 Homolog, Protein Translocation Regulator gene
SER	Smooth Endoplasmic Reticulum
SIA	Supraintestinal Artery
SIV	Subintestinal Vein
TALEN	Transcription Activator-Like Effector Nucleases
TRP	Transient Receptor Potential
TRPP2	Transient Receptor Potential Polycystic-2
V2R	Vasopressin Receptor-2
VEGF	Vascular Endothelial Growth Factor
WES	Whole-Exome Sequencing
Wnt	Wingless-related integration site
WSC	cell Wall integrity and Stress response Component
WT	Wild-Type
ZFN	Zinc Finger Nucleases

1. INTRODUCTION

In today's world, advances in medicine and research studies, allowed many chronic diseases to be manageable and livable. World Health Organization predicted that, by 2020, about 57% of the people in the world would be affected by a chronic disease ¹ and many of them will get to live to their old age. However, these diseases resonate differently when we take into consideration the quality of life. Particularly in cases for which there is yet no specific treatment and when there is degeneration.

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is an inherited chronic kidney disease that affects over 12 million people worldwide in all ethnic groups and one of the contributing factors for the increasingly large share of global disease burden due to the disability it causes ^{2,3}.

It has been estimated that, over an 80-year lifetime, 1 in 400-1000 people are at risk of being affected with ADPKD ⁴. Recent population-based whole-genome sequencing data supported this rate, showing that protein-truncating and clinically confirmed mutations constitute a lifetime risk of ADPKD for 9 in 10,000 individuals ⁵. However, considering age-dependent penetrance, complex genetics and incomplete clinical determination of ADPKD, some recent epidemiological studies of the European Union have estimated lower rates of ADPKD prevalence (1 in more than 2000 individuals) ⁶. Nonetheless, this disease is the most common genetic cause and a leading cause of kidney failure ^{7,8}.

This disease is clinically characterized by the development of several fluid-filled kidney cysts that increase in number and size throughout the patients' life. As these cysts develop, they disrupt the normal renal architecture and gradually destroy healthy renal parenchyma, leading to kidney enlargement (Figure 1.1.), abdominal pain, cyst hemorrhage, hematuria, nephrolithiasis, cyst infections and, eventually, renal failure ^{2,9}.

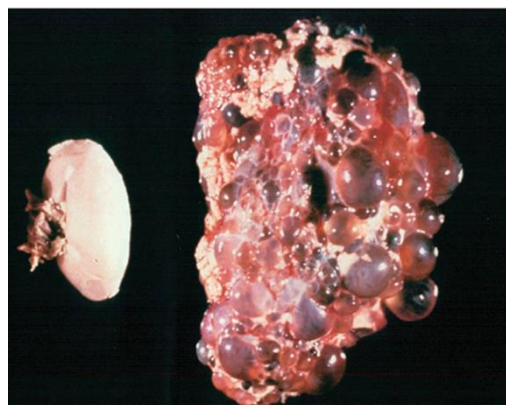


Figure 1.1 – ADPKD kidney (right) with several fluid-filled cysts compared with normal kidney (left) (image adapted from ¹⁰).

Despite continuous destruction of renal parenchyma, the renal function is maintained within the normal range for decades, due to compensatory hyperfiltration in healthy glomeruli. Therefore, by the time that there is a decline in glomerular filtration rate (GFR) (usually after the fourth decade of life) the majority of the nephrons have been destroyed ². Extrarenal manifestations include cysts in other organs, such as the liver, seminal vesicles, pancreas and arachnoid membrane, hypertension, as well as other abnormalities, such as intracranial aneurysms and dolichoectasias, cardiac valvular abnormalities, aortic root dilation and aneurysms, and abdominal hernias ¹¹.

1.1. Genetics

ADPKD is most commonly caused by mutations in *PKD1* (OMIM-601313) and *PKD2* (OMIM-613095) genes which account for the majority of ADPKD cases (78% and 15% of cases, respectively)¹².

PKD1 is located at chromosome region 16p13.3 and is a large complex gene with 46 exons spanning over 52 kb of genomic DNA that encodes a 14 kb transcript. The entire 5' region of *PKD1* up to exon 33 is duplicated at six other sites on chromosome 16. These homologous pseudogenes (with ~95% homology to *PKD1*), with many of them encoding messenger RNA (mRNA) transcripts (which are not translated), make it difficult for mutation detection, as only the 3' 3.5 kb of the *PKD1* transcript is single copy^{9,13,14}. In its turn, *PKD2* is situated at chromosome region 4q21 and, unlike *PKD1*, has no known pseudogenes and encodes a 5.3 kb mRNA with 15 exons^{9,15,16}.

ADPKD shows both *locus* and allelic heterogeneity with more than 1500 different pathogenic mutations of *PKD1* and *PKD2* identified and reported on the ADPKD mutation database (<https://pkdb.mayo.edu/>)^{17,18}. As such there is a widespread interfamilial and intrafamilial variability in age-dependent penetrance and clinical disease severity^{5,9,11}.

Accordingly, *PKD1* mutations are associated to a more severe disease than *PKD2* mutations. These patients have earlier-onset End-Stage Renal Disease (ESRD), with median age of 58.1 years in *PKD1* versus 79.7 years in *PKD2*, lower GFR and larger kidneys^{5,17,19}.

There is also a significant allelic effect in *PKD1*. Recent studies found a strong association between renal disease severity and the type of mutation: a milder disease is associated with non-truncating compared with truncating mutations^{17,20,21}. This indicates that a considerable proportion of non-truncating *PKD1* mutations are incompletely penetrant (hypomorphic), meaning the mutant protein has some residual function, resulting in later-onset ESRD than with more penetrant alleles^{17,22}.

Besides *PKD1* and *PKD2* genes, two additional ones were recently found to be linked to cases of ADPKD. In 2016, after sequencing genetically unresolved families with autosomal dominant polycystic kidney or liver disease, Porath and her team identified heterozygous mutations in *GANAB* (catalytic α subunit of glucosidase II encoding gene) (OMIM-104160). This gene is located at the chromosomal region 11q12.3, has a genomic size of 21.9 kb and is accountable for about 3% of previously genetically unresolved ADPKD cases. The renal phenotype associated with *GANAB* mutations is apparently even milder than that of *PKD2*, without renal insufficiency and kidney enlargement due to a few large cysts²³.

Furthermore, due to the use of whole-exome sequencing (WES) and next-generation sequencing (NGS), it was possible to find an additional gene associated to ADPKD: *DNAJB11* (OMIM-611341). This gene is located in chromosome region 3q27 with a coding segment extending over 15.1 kb of genomic DNA and is the cause for about 1.2% of genetically previously unresolved ADPKD-affected families²⁴. Mutations in *DNAJB11* account for an atypical phenotypic presentation of the disease characterized by normal-sized cystic kidneys and progressive interstitial fibrosis, which results in late-onset ESRD²⁴.

Some cases of ADPKD remain genetically unresolved due to missed variants of the existing genes (including detected variants indeterminate for pathogenicity), deep intronic cryptic splicing changes and mutations in promoter and 3'UTR regions; as well as mosaic, *de novo* mutations that can be associated with milder ADPKD or even mutations in other PKD genes (*HNF-1 β* , *PRKCSH* and *SEC63*) that can present a similar phenotype^{12,23,25–27}.

1.2. ADPKD Proteins

1.2.1. The PC1/PC2 complex

Polycystin-1 (or PC1) is the product of *PKD1*, the gene that when mutated is responsible for the majority and more severe cases of ADPKD. It consists of a large transmembrane protein of 4303 amino acids (aa) (460 kDa peptide backbone, ~600 kDa after glycosylation), with 11 transmembrane domains, an extensive extracellular N-terminal region of ~3000 aa and a short cytoplasmic carboxyl-terminal portion (197 aa) that includes a coiled-coil region that mediates interaction with PC2 (Figure 1.2.)^{13,15,28}.

The ectodomain of this protein consists of several motifs, such as Leucine-rich repeat (LRR) domain and C-type lectin and cell wall integrity and stress response component (WSC) domain, involved in protein-protein and protein-carbohydrate interactions, respectively. Its immunoglobulin domain-like region of 16 PKD repeats has been shown to have mechanical stability properties which may also be the part for repeats in the receptor for egg jelly (REJ) module²⁹⁻³¹.

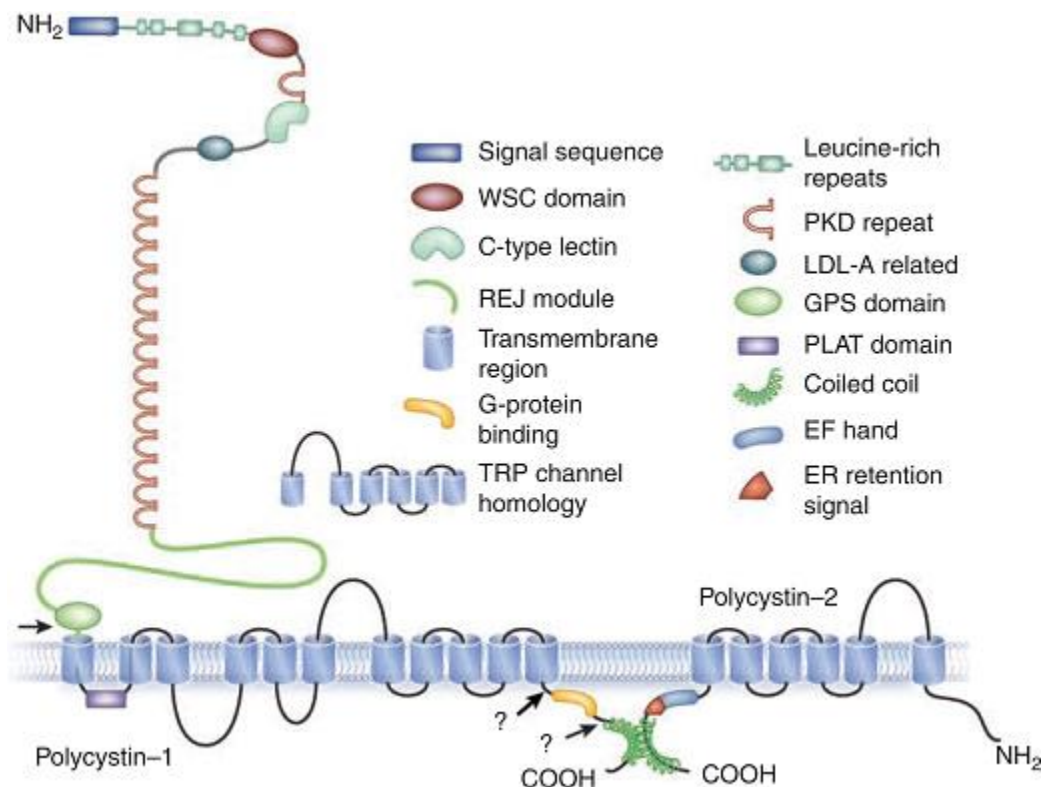


Figure 1.2. – Schematic representation of Polycystin-1 and Polycystin-2, their domains and interaction via C-terminal tails. Black arrows point to PC1 cleavage sites. (adapted from³²). WSC – cell-wall integrity and stress response component; REJ – Receptor egg jelly; TRP – Transient receptor potential; PKD – Polycystic kidney disease; LDL-A – Low density lipoprotein A; GPS – G protein-coupled receptor proteolytic site; PLAT – Polycystin-1, Lipoxigenase, Alpha-Toxin; ER – Endoplasmic reticulum.

A major processing event that gives rise to functional PC1 is *cis*-autoproteolysis cleavage at the G protein-coupled receptor proteolytic site (GPS) in the early secretory pathway, yielding a large N-terminal and a transmembrane C-terminal product, which remain tethered noncovalently^{33,34}. The process is fundamental for complete functional activation of PC1, namely its folding, maturation, and surface localization³⁵⁻³⁷. In fact, studies have shown that some *PKD1* missense mutations disrupting this cleavage impair the ability of PC1 to induce tubulogenesis, with Madin-Darby Canine Kidney

(MDCK) cells and *PKD*^{V/V} mice forming cyst-like structures instead of tubules as seen in wild-type (WT) controls^{33,34}.

PC1 exists however as a heterogeneous population of full-length and cleaved forms, and it is thought that the uncleaved form may play a critical role during development. Indeed, to investigate the role of PC1 cleavage *in vivo*, a group of investigators created, by gene targeting, a *PKD1* knockin mouse (*PKD1*^{V/V}) that expressed noncleavable PC1 and reported that *PKD1*^{V/V} mutants overcame embryonic lethality but developed rapidly progressive PKD. When characterizing the cleavage pattern of endogenous PC1 in the wild-type (WT) and *PKD1*^{V/V} mice, they discovered that PC1 is ubiquitously and incompletely cleaved in WT mice, so that both forms coexist. This data suggested a critical but restricted role of cleavage for PC1 function and a differential function of the two types of PC1 molecules *in vivo*³⁴.

It has also been suggested that the C-tail of PC1 may also undergo cleavage (question marks in Figure 1.2), generating products that translocate to the nucleus and act as coactivators of several transcriptional pathways^{38,39}

PC1 is widely expressed in epithelial cells of developing and mature renal tubules, as well as in several other somatic tissues such as heart, liver, bone and endocrine glands⁴⁰. It is more specifically localized at the primary cilia membrane, a sensory organelle found on the surface of the former mentioned cells, but it can have other subcellular localizations, including at tight junctions, adherens junctions and desmosomes of basolateral membrane, focal adhesions and apical vesicles^{15,41}. However, there are differences in PC1 expression during development, with higher expression of PC1 in human fetal renal tissues, during the development of the embryo, than in adult tissues, subsequent to a significant down-regulation shortly after birth⁴².

It is thought that PC1 functions as a receptor that senses extracellular chemical and mechanical force stimuli and regulates cytosolic cyclic adenosine monophosphate (cAMP) intracellular levels and its downstream signaling⁴³. It may also be implied in cell-cell interactions and cell-matrix adhesion as it is part of cell adhesion complexes⁴⁴⁻⁴⁷.

Another determinant protein in ADPKD is polycystin-2 (PC2), a protein of 968 aa (~110 kDa) that comprises two intracytoplasmic tails, both N and C termini, and six transmembrane domains⁴⁸. The transmembrane region of PC2 has homology with the last six transmembrane domains of PC1 (Figure 1.2.). The C-terminal tail comprises an EF hand (helix-loop-helix motif) with a calcium-binding pocket, an endoplasmic reticulum (ER)-retention signal, and two coiled-coil domains, one of which is implicated in the interaction with PC1⁴⁹⁻⁵¹. Naturally occurring truncating mutations that remove this C-terminal domain, and thus likely disrupt or halt its interactions and regulatory functions, are sufficient to cause ADPKD⁴⁸.

Unlike PC1, PC2 expression levels do not change as strikingly during development but its pattern is similar with that of PC1¹⁶. PC2 is mainly expressed during the development of epithelial cells of proximal and distal renal tubules, but also in developed structures of the pancreas, liver, lung, bowel, brain, reproductive organs, placenta and thymus^{52,53}. Other cell types such as skeletal muscle, myocardial cells, neurons and vascular endothelium and smooth muscle cells also express both PC1 and PC2, the latter suggesting that ADPKD vascular abnormalities may be related to the disruption of these proteins⁵². At the subcellular level, PC2 is mainly localized in the endoplasmic reticulum (ER) and early Golgi, but it can also be found at the plasma membrane, mitotic spindles, lamellipodia, and primary cilium^{40,54,55}.

PC2, also known as TRPP2, shares topologic and sequence homology with the transient receptor potential (TRP) family of calcium-responsive cation channels¹⁵ and, therefore, functions as a high-conductance, nonselective, calcium-permeable channel^{48,56,57}. PC2 function may vary given its subcellular location, but it is mainly responsible for regulating intracellular Ca^{2+} levels⁴³. It responds to local increases in Ca^{2+} concentration, but also to changes in pH, voltage or phosphorylation status, releasing Ca^{2+} from intracellular stores, since it acts as a Ca^{2+} -release channel on the endoplasmic reticulum^{40,43,58}.

This protein also interacts with other main Ca^{2+} channels in the ER such as: ryanodine receptor (RyR), which mediates calcium-induced calcium release (PC2 inhibits RyR function by binding the channel in its open state and decreasing its conductance); and inositol-1,4,5-triphosphate receptor (IP3R), a Ca^{2+} channel activated by inositol triphosphate (IP3), binding of the PC2 C-terminal domain to IP3R alters IP3-induced Ca^{2+} flux^{40,59}.

Furthermore, PC2 interacts and forms complexes with several other proteins and cytoskeletal components such as actin and microtubules, which has been shown to be of importance for its channel expression, function and involvement in sensing hydrostatic and osmotic pressure^{45,60}.

Most importantly though, PC2 forms a mechanosensory complex with PC1 at the membrane of primary cilia in the renal epithelium (Figure 1.3.) that is essential for Ca^{2+} homeostasis and consequently for the differentiation and maintenance of renal epithelium^{61,62}. Although the precise structure and function of PC1/PC2 complex is yet unresolved, it is postulated that PC1 senses urine-flow that bends the cilium and transduces it into a ciliary Ca^{2+} influx through PC2. This Ca^{2+} wave is propagated and amplified in the cytoplasm through the release of more Ca^{2+} from ER storages, a process that depends on PC2 expressed in this organelle^{61–64}. Disruption of these proteins, as in ADPKD cells, leads to a decrease in basal intracellular Ca^{2+} levels which will then dysregulate several signaling pathways and initiate cystogenesis^{65,66}. However, the lack of mechanically induced Ca^{2+} increases in primary cilia has been reported, therefore this hypothesis is still uncertain⁶⁷.

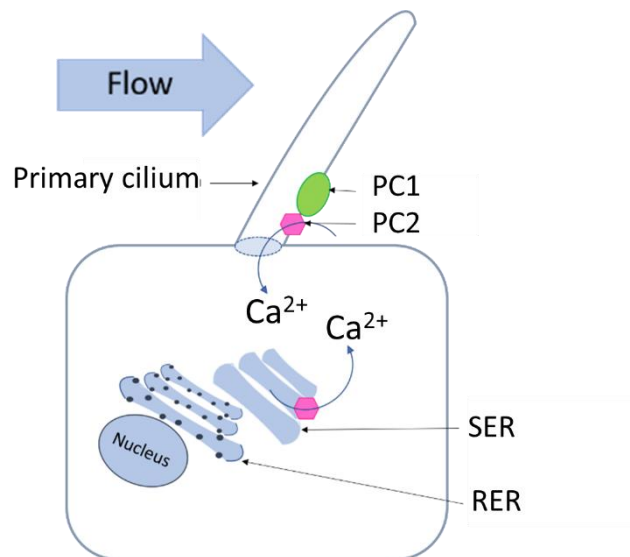


Figure 1.3. – Schematic representation of a tubular epithelial cell. PC1/PC2 as a mechanosensory complex located at the membrane of the primary cilium of kidney epithelial cells. Urine flow sensed by PC1, Ca^{2+} influx through PC2 and release of Ca^{2+} from cellular stores at the Endoplasmic Reticulum in cytoplasm. SER- Smooth Endoplasmic Reticulum; RER – Rough Endoplasmic Reticulum (adapted from¹¹).

1.2.2. α -Glucosidase II and DNAJB11

The other two proteins that are involved in rare cases of ADPKD, α -Glucosidase II and DNAJB11, have something in common: when mutated they disrupt efficient maturation and trafficking of transmembrane proteins, especially PC1, which results in renal or liver cystogenesis¹².

GANAB encodes the catalytic α subunit (GII α) that, together with a regulatory β -subunit (GII β), encoded by *PRKCSH*, forms glucosidase II: a resident enzyme of the ER involved in N-linked glycosylation. Which is a quality control process fundamental for proper folding, maturation and trafficking of transmembrane glycoproteins^{23,68}. GII α has 944 aa (~107 kDa) and is expressed in several tissues such as the nervous system, liver, intestine, bone marrow, lung, and the kidney^{69,70}.

It has been shown that mutations in *GANAB* and, therefore, loss or reduction of functional GII α disrupts the maturation and localization of PKD proteins (PC1 and PC2) to the cell surface and cilia, being responsible for some cases of ADPKD²³.

Likewise, DNAJB11 is involved on the proper folding, trafficking and degradation of membrane or secretory proteins^{24,71,72}. This glycoprotein is composed by 358 aa, with approximately 4.1 kDa, that contains a highly conserved J domain of about 70 aa with a characteristic His-Pro-Asp (HPD) motif⁷³. Localized on the ER lumen, DNAJB11 is one of the most abundant co-chaperones of the binding immunoglobulin protein (BiP), a heat shock protein chaperone required for the proper folding and assembly of proteins at the ER²⁴. And, as such, this protein showed great potential to be of importance for ADPKD.

Indeed, when evaluating if DNAJB11 loss was associated with impaired maturation and localization of PC1, it was showed that the mature, cleaved N-terminal PC1 was less abundant by >50% in *DNAJB11*^{-/-} cells than WT along with elevated levels of full length and immature PC1 in *DNAJB11*^{-/-} clones; and the PC1 membrane expression in *DNAJB11*^{-/-} cells was less than half than that seen in WT cells²⁴.

Given these recent findings, further research into these protein maturation disorders might allow further insight into undetected variations of ADPKD and help the development of specific therapeutic approaches with the purpose of restoring the proper folding and expression of PC1 in the cilia membrane⁷⁴.

1.3.Cystogenesis

Despite ADPKD being a dominantly inherited disease and each renal tubular cell a carrier of a germline mutation, not all nephrons develop cysts. In fact, cysts are focal lesions and only 1-2% of nephrons develop them ^{7,75}. There are two main explanations that have been theorized to explain this phenomenon.

The first mechanism that was proposed was a “two hit hypothesis”, that postulated that, besides the germ line mutation, the tubular cells needed to suffer a somatic mutation or “second hit” of the remaining wild-type allele, inactivating it (Figure 1.4.). The polycystin deficient cells then begin clonal expansion and initiate cyst formation and enlargement, which explains the focal quality of the cysts ⁷⁶.

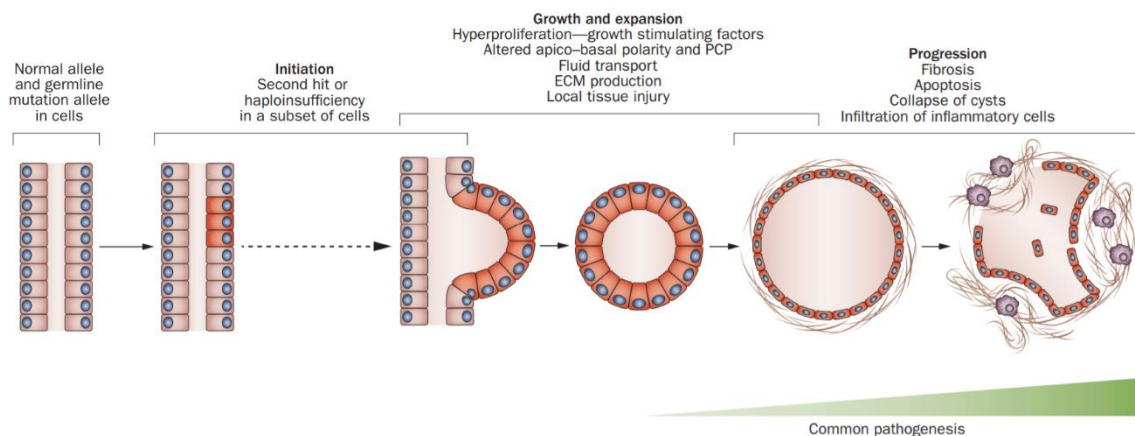


Figure 1.4. – Cystogenesis mechanism representation of both two-hit and haploinsufficiency hypothesis, and further process of cyst growth and expansion, culminating in tissue alterations to cystic epithelium which characterize ADPKD pathogenesis (adapted from ⁷⁷)

Corroborating this theory, analysis of cystic epithelia from both *PKD1* and *PKD2* associated ADPKD kidneys showed independent somatic mutations, which resulted in loss of heterozygosity or base-pair changes, in a large portion of renal cysts ^{78–82}. The same was verified in mouse models that, together with an unstable *Pkd2* allele that underwent somatic mutations resulting in a functionally null allele and a germ line null, developed polycystic kidney and liver disease ⁸³.

This hypothesis, however, has some uncertainty given the high number of individual cysts, which would imply a very high amount of somatic mutations. Various studies also demonstrated that cysts may develop with some level of polycystin present ^{84,85}. Which leads to the following premise that is more likely to explain cystogenesis: haploinsufficiency or dose effect. This theory postulates that the reduction of polycystin signaling below a critical threshold level is sufficient to develop a cystic phenotype ^{84–86}. Thus, when the level of functional PC1 or PC2 decreases below the critical threshold necessary to maintain normal tubulogenesis, associated with environmental factors, physiologic demands, somatic mutations or stochastic factors, cystogenesis takes place ^{87,88}. It was first suggested when Lantinga and colleagues observed that a mouse model that only expressed 13-20% of normal *Pkd1* levels presented a cystic phenotype plus cardiovascular abnormalities, which is a pathogenic feature similar to human ADPKD ⁸⁴.

Some studies have also linked the timing of polycystin loss to rapidity of cyst formation and progression of the disease ^{89,90}.

At the cellular level, cystogenesis comprises two main processes that include cell proliferation and fluid secretion into the lumen of the cyst, leading to the isolation and inflation of the cyst from the nephron. Which involves the disruption of several signaling pathways such as intracellular Ca^{2+} , cAMP, mammalian target of rapamycin (mTOR) and the planar cell polarity (PCP) that will be briefly clarified ⁹¹.

1.3.1. The essential second messengers: Ca^{2+} and cAMP

As was mentioned before, the PC1/PC2 complex, located at the membrane of primary cilium in the renal epithelium, is essential for Ca^{2+} homeostasis and consequently the differentiation and maintenance of renal epithelium, as it translates flow into a ciliary Ca^{2+} signal further amplified in the cytoplasm. The disruption of these proteins leads to a decrease in basal intracellular Ca^{2+} levels which dysregulates several other pathways such as cAMP. Intracellular levels of cAMP are determined through its synthesis from adenosine triphosphate (ATP) by adenylate cyclase (AC) and degradation via hydrolysis, mediated by phosphodiesterases ⁹².

Several PKD models have shown that cAMP levels are elevated, which is very relevant as this second messenger is implicated in cell proliferation, differentiation and guiding of the secretion of fluid ^{32,93,94}.

The role of cAMP on proliferation in ADPKD cells is mediated by protein kinase A (PKA) stimulation of the mitogen-activated protein kinase/extracellular regulated kinase (MAPK/ERK) pathway ⁹⁵. This pathway is activated through a succession of phosphorylations to PKA, B-Raf and MAPK, promoting cell proliferation ^{96,97}.

Although the mechanisms underlying the higher levels of cAMP are not yet fully known, it is believed that it may involve an imbalance in the production and degradation of cAMP. The reduction of Ca^{2+} levels in the cytoplasm, may stimulate the AC and inhibit phosphodiesterases that are Ca^{2+} -dependent. Therefore, increasing cAMP synthesis and inhibiting its hydrolysis ^{98,99}.

However, one of the greatest contributors to elevated levels of cAMP is the vasopressin pathway. Vasopressin is a hormone that is released in response to dehydration or decreased blood volume, causing vasoconstriction and stimulating the retention of water, which occurs mainly in the collecting duct and distal tubule of the kidney ¹⁰⁰. The impairment of urine concentration, which is one of the earliest ADPKD manifestations, seems to be the cause of the increased vasopressin pathway ¹⁰¹. The binding of vasopressin to the vasopressin receptor-2 (V2R) leads to the increase of cAMP levels (vasopressin is an agonist of AC), which then activates PKA. PKA in turn targets and phosphorylates the water channel aquaporin 2 (AQP2), which is translocated to the apical cell surface, and the epithelial sodium (Na^+) channel (ENaC), increasing water permeability and Na^+ reabsorption ¹⁰².

Consistently, several studies have associated increased levels of copeptin, a vasopressin surrogate that allows to infer its circulating concentration, with disease severity and progression in ADPKD ¹⁰³. Furthermore, the expression of V2R and AQP2 is increased in cystic kidney cells and the use of V2R antagonists lowered renal cAMP, inhibited cyst development and slowed disease progression ¹⁰⁴. Indeed, Tolvaptan, which acts as a V2R antagonist, lowering intracellular cAMP levels of cyst lining cells, is effective in decelerating disease progression and is the only pharmaceutical

approved for ADPKD management. However, it leads to heavy aquaretic side effects, which limits eligible patients and life-long treatment ^{105,106}.

Besides the channels already talked about, PKA also phosphorylates the cystic fibrosis transmembrane conductance regulator (CFTR), the ion channel that when mutated causes Cystic Fibrosis ¹⁰⁷. This allows the flow of chloride (Cl⁻) ions by an electrochemical gradient, which plays an important role in Cl⁻ driven transepithelial fluid secretion, responsible for cyst inflation in ADPKD ¹⁰⁸. Indeed, fluid accumulation within ADPKD cysts involves CFTR-like Cl⁻ currents ¹⁰⁹. Besides CFTR activation, it also seems that healthy individuals have low levels of CFTR in kidney cells, compared with ADPKD cyst-lining cells which have higher levels of CFTR apical expression ^{109,110}.

Altogether, everything points to the idea that high levels of cAMP are associated with cyst and ADPKD progression, which makes this messenger as its pathways one of the main targets of therapeutic trials.

1.3.2. Growth and survival: mTOR

The mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that senses cellular states of energy, stress, nutrition, oxygenation and other factors, in order to regulate cellular pathways that control cell growth and survival, such as metabolism, protein translation, autophagy and proliferation ¹¹¹. Overall, in the presence of certain growth conditions, mTOR signaling is activated to coordinate nutrient import and macromolecular synthesis, leading to the increase of cell size and mass ¹¹².

Under normal conditions, *in vitro* studies indicate that PC1 inhibits the mTOR pathway through its C-terminal tail that is cleaved and released into the cytosol. Therefore, the disruption of this interaction in ADPKD leads to the upregulation of the mTOR pathway in cyst lining epithelial cells ^{113,114}. In some studies using rodent models, investigators were able to reduce cyst size and kidney volume by using mTOR inhibitors sirolimus (rapamycin) and everolimus ^{115,116}.

1.3.3. Cell orientation: Noncanonical Wnt signaling

Wnt signaling comprises a group of signal transduction pathways, in which extracellular Wnt glycoproteins signal by activating cell surface receptors, with two main pathways: β -catenin-dependent (or canonical) and noncanonical ⁹¹. It is postulated that PC1 C-terminal tail associates with β -catenin, inhibiting canonical Wnt signaling, a pathway which becomes activated in ADPKD cyst cells ¹¹⁷.

On the other hand, noncanonical Wnt signaling regulates planar cell polarity (PCP), which aligns cells orientation in an appropriate direction and position in order to maintain normal cell function. Renal tubular cells normally divide parallel to the nephron lumen, allowing the tubule to extend lengthwise, but in ADPKD, disruption in Wnt signaling, causes aberrant orientation of cell division and may contribute do cyst formation ⁹¹.

In summary, cystic epithelium will present several pathologic traits including increased proliferation, increased apoptosis, remodeling of extracellular matrix, a secretory phenotype, dysregulated metabolism and aberrant planar cell polarity.

1.4. Renal manifestations

ADPKD cysts arise as saccular outpouchings from any nephron segment (Figure 1.5.) but the majority of them originate from the collecting ducts ⁸⁴. It is thought that injured tubules and expanding cysts release inflammatory cytokines leading to macrophage infiltration, fibrosis, and neovascularization, altering healthy parenchyma and the impairing remaining renal function ⁷.

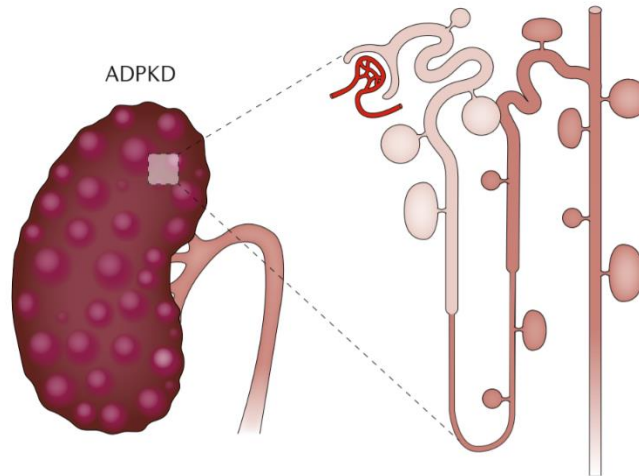


Figure 1.5. – Nephron representation from an ADPKD kidney, showing the different segments from which the cysts can originate (adapted from ¹⁹).

At first, hypertrophy of functional nephrons and glomeruli compensate parenchymal loss and maintain GFR. Therefore, this parameter is not a good method to monitor ADPKD progression ¹¹⁸. Instead, total kidney and cyst volume, measured by imaging techniques (ultrasound, CT and NMR scans), have been shown to be better predictors of progression of disease, when monitoring ADPKD, as it allows to infer renal function decline ¹¹⁸.

As ADPKD progresses other complications appear as hematuria, pyelonephritis, nephrolithiasis, abdominal pain and, potentially, renal failure.

1.5. Extrarenal manifestations

As we have noticed, ADPKD is a systemic disease that affects several organs and systems, that is not limited to kidney cysts. Moreover, polycystic liver disease (PLD) is the most common extrarenal manifestation. Hepatic cysts are formed due to excessive proliferation and dilation of biliary ductulus and peribiliary glands ³². The growth of liver cysts is promoted by estrogens, growth factors and cytokines, therefore cyst prevalence and volume is higher in women than men ^{119,120}.

Although they are rare in children, their frequency increases with age and it can have a prevalence as high as 58% between 15-24 years old ¹²¹. Usually this disease is asymptomatic, but as the lifespan of patients with ADPKD was extended with dialysis and transplantations, symptoms related to cyst expansion or other complications became more frequent ³².

Some patients also develop cysts in other organs such as the pancreas (5%) and arachnoid membrane (8%), often asymptomatic. The former are very rarely associated with recurrent pancreatitis and cancer, whilst the latter increase the risk of subdural hematomas ³². With a higher incidence, affecting 50% of men, seminal vesicle cysts rarely cause infertility, even though men with ADPKD exhibit sperm abnormalities and defective motility ³².

Additionally, ESRD patients with ADPKD frequently have colonic diverticulosis and diverticulitis compared with those with other renal diseases ¹²².

1.6.Hypertension

Hypertension is the most common feature of ADPKD and one of the major contributors for disease progression, kidney enlargement, and cardiovascular complications, representing an important risk factor for ESRD ¹²³. Affecting more than 60% of adult individuals, it occurs long before loss of kidney function ¹²⁴. In fact, it can be present already in childhood, affecting 20-35% of children with ADPKD ^{125,126}. In a 2008 study, hypertensive and borderline hypertensive children already had a significantly increased left ventricular mass than normotensive children, suggesting it develops early in ADPKD ¹²⁷. Such organ damage is a risk factor for cardiovascular disease, the most common cause of death in these patients ¹²⁸.

It is known the association between the severity of kidney disease and hypertension: hypertensive ADPKD patients present higher kidney volumes, greater rate of increase in kidney volume, increased proteinuria and decreased renal blood flow than normotensive ADPKD patients ¹²⁴. Therefore, it is important to accompany children with positive family history of ADPKD to assure and early detection and control of hypertension.

An important unresolved question in the field is whether hypertension is primarily caused by abnormal endothelial/vascular function, or it is secondary to an abnormal activation of the renin-angiotensin aldosterone system (RAAS), or both.

In normal physiological conditions, within afferent arterioles of the kidney, juxtaglomerular (JG) cells contain prorenin. Decreased blood pressure, β -activation, or decreased Na^+ concentration in the distal convoluted tubule cause the activation of these cells, which leads to cleavage of prorenin to renin ¹²⁹. When released into the blood, renin acts to cleave angiotensinogen into angiotensin I. Present in the vascular endothelium of the kidneys, angiotensin converting enzyme (ACE) converts angiotensin I to angiotensin II. This hormone acts on the cortex of adrenal gland, proximal convoluted tubule of the kidney, systemic arterioles and brain, which stimulates aldosterone, increases Na^+ reabsorption, causes arteriolar vasoconstriction and increases water reabsorption in the kidney. Higher levels of Na^+ in the body (which rise blood osmolarity), increases in blood volume, and vasoconstriction (which increases peripheral resistance), lead to an increase in blood pressure ¹³⁰.

In ADPKD patients, there are two main processes involved in hypertension development. In one hand, as renal cysts enlarge, they compress renal vasculature causing intra-renal ischemia. As blood flow is decreased to parts of the kidney, JG cells interpret as though blood pressure is low, triggering the secretion of renin and activating the RAAS system we already explained ¹²⁴. With increasing cyst size, more intra-renal RAAS activation occurs perpetuating hypertension.

On the other hand, decreased or absent polycystins expression in endothelium and vascular smooth cells lead to a decrease in Ca^{2+} levels which results in reduced nitric oxide (NO) release (endothelial NO synthesis activity is Ca^{2+} dependent) and to an increase in cAMP levels ¹³¹. Low levels

of NO alter the endothelial response to shear stress, resulting in an attenuation in vascular relaxation, inducing hypertension. In renal vasculature, low levels of NO can also activate local oxidative stress pathways, activating local/intra-renal RAS ¹²⁴. Indeed, human cystic epithelium has been shown to produce renin, angiotensinogen (precursor of angiotensin I), ACE and angiotensin II ¹³².

1.7. Cardiovascular abnormalities

The process by which PC1 and PC2 regulate cardiovascular development and functions is yet to be fully understood. It is known that polycystins are expressed in vascular endothelial cells and smooth muscle cells and are involved in cell-matrix connectivity. Primary cilium of endothelial cells, regulate blood vessel function through blood flow sensing, cell migration, and Ca^{2+} and NO signaling ^{133,134}. And it has been demonstrated that, in these cells, fluid shear stress induces activation of PC2, triggering Ca^{2+} signaling and increasing the biosynthesis of intracellular NO, through activation of endothelial nitric oxide synthase (eNOS) via protein kinase C (PKC) and calmodulin (CaM), which results in smooth muscle dilatation and flow-induced vascular relaxation, ^{63,135}. Furthermore, PC2 has also been implicated in sensing volume pressure in vascular smooth muscle cells and myogenic response in mesenteric and cerebral arteries, through Ca^{2+} homeostasis ^{136,137}.

However, several studies report differing information for polycystins' involved pathways and responses and so we have to consider that most likely the regulation and function of polycystins is not homogenous for all blood vessels and findings in one arterial bed must not be translated to other vasculature ¹³⁸.

Nonetheless, it is understood that the impairment of polycystins expression leads to enhanced vascular smooth muscle contractility and impaired endothelial-dependent vasorelaxation, hypertension, extracellular matrix changes and inflammation ¹³⁹, all of which cause the cardiovascular manifestations of ADPKD: intracranial aneurysms and dolichoectasias, aortic and cervicocephalic artery dissections, coronary artery aneurysms, aortic insufficiency and regurgitation, mitral valve prolapse and left ventricular hypertrophy ³². The next chapters will focus on the most common and significant.

1.7.1. Intracranial aneurysm

Intracranial aneurysm (ICA) is a very dangerous complication of ADPKD. It consists of an outpouching of a brain blood vessel, which can leak or rupture. The majority of ICA are small, with less than 6 mm in diameter, and occur mainly in the middle cerebral artery, internal carotid artery and anterior communicating artery (Figure 1.6.) ¹⁴⁰.

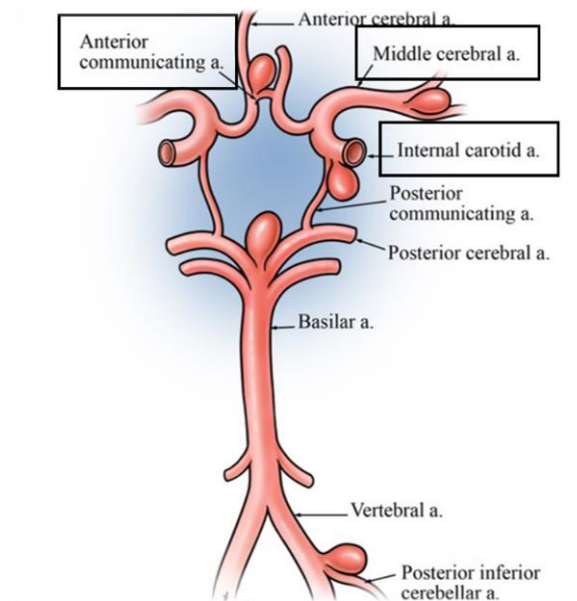


Figure 1.6. – Illustration showing most common sites for intracranial aneurysm development in ADPKD: middle cerebral artery, internal carotid artery and anterior communicating artery (adapted from ¹⁴¹).

The rupture of an ICA in ADPKD patients is associated with a mortality of 60% and 50% of survivors are left with neurological deficits ^{142,143}. The average age of rupture, at 41 years old, is similar to that reported in other familial forms of ICA, but 10 years earlier than sporadic ICAs ¹⁶. A ruptured ICA results in a subarachnoid hemorrhage (SAH) that is associated with a sudden and intense headache ¹⁴⁴.

Even though its occurrence is not frequent (5%–9 %), it is 3-5 fold more often than among general population ¹⁴². However, the prevalence is higher in patients with a family history of an ICA (16%), demonstrating that genetic factors are important in the etiology ³².

In fact, it is thought that ADPKD associated ICAs are caused by alterations in the vasculature, directly related to mutations in *PKD1* or *PKD2*. While normal vascular smooth muscle cells provide collagen synthesis and maintenance of a healthy extracellular matrix ¹⁴⁵, in ADPKD, polycystins haploinsufficiency changes vasculature and potentiates the formation of ICAs due to dysregulated pathways that lead to hypertension, extracellular matrix changes and inflammation ¹³⁹. Studies with *Pkd2*^{+/-} mouse models demonstrated that *Pkd2*^{+/-} vascular smooth muscle cells exhibit increased rates of proliferation and apoptosis, and, when hypertensive, individuals had an increased susceptibility to vascular injury and premature death ^{136,146}.

1.7.2. Cardiac abnormalities

Many ADPKD patients (26%) also develop mitral valve prolapse (MVP), a condition where the two valve flaps of the mitral valve do not close properly ¹³⁹. This might cause blood to leak back into the left atrium, which is called regurgitation, producing a heart murmur. Other alterations associated with this defect are palpitations and chest pain not associated with exercise or exertion ¹³⁹.

It is yet unclear the pathophysiology underlying this condition. However, the examination of affected valvular tissue revealed defective collagen synthesis and myxomatous degeneration ¹⁴⁷. Some

investigators also speculated that mitral regurgitation could be prompted by the hypertension, since it causes left ventricular hypertrophy ¹⁴⁸.

Left ventricular hypertrophy (LVH) is very associated with hypertension in ADPKD and is a powerful independent risk factor for cardiovascular morbidity and mortality ¹⁴⁹. It has also been related with poor renal and overall outcomes in these patients ¹⁵⁰. However it seems that hypertension is not the only factor that leads to the development of LVH and other studies have reported its occurrence in normotensive patients ¹⁵¹.

1.8.Zebrafish: an ADPKD model

Several ADPKD models are available to study ADPKD, including genetically engineered *Pkd1* ^{152,153} or *Pkd2* ¹⁴⁷ mutant rodent models and *in vitro* mammalian cells such as MDCK ¹⁵⁴ and Human Embryonic Kidney Cells 293 (HEK293) ¹⁵⁵. These models are very useful to study cyst development and progression ^{90,156,157}, the role of primary cilia ^{90,158}, the involved disrupted signaling pathways ^{159–161} and preclinical tests in search of effective therapies ^{162,163}. However, mouse models limit live observation and mammalian cells the study of whole organism extrarenal manifestations related to ADPKD such as vascular anomalies.

Therefore, we decided to use zebrafish (*Danio rerio*) as animal model to study vascular anomalies associated with ADPKD. Indeed, this animal has been characterized as an important model system to study vascular development and human diseases *in vivo* ^{164,165}. Unlike other vertebrate models, adult zebrafish are small (4-5 cm in length), develop quickly, reaching sexual maturity at 3 months old and have an easy and low-cost maintenance ¹⁶⁶. Similar to other fish species, zebrafish have high fecundity, with each reproductively active couple reaching 300 eggs/week ¹⁶⁷.

Moreover, they are particularly advantageous, because fertilization and development occur externally, and embryos have optical transparency, which allows high-resolution of internal imaging of the developing animal ¹⁶⁵. This transparency can be further prolonged through larval stages by incubating zebrafish embryos, after 24 hours post-fertilization (hpf), in 200 μ M 1-phenyl-2-thiourea (PTU) solution which avoids the development of pigmentation ¹⁶⁸. Besides this, zebrafish have a closed circulatory system, and anatomical features and molecular mechanisms of vessel formation and development are very similar to those in humans and other higher vertebrates ¹⁶⁵.

Curiously, zebrafish have several duplicated genes, which resulted from a whole genome duplication that occurred after their divergence from the tetrapod's lineage ¹⁶⁹. Despite this, usually zebrafish chromosomes are mosaically orthologous to human chromosomes ¹⁷⁰. In fact, zebrafish have several *pkd* genes (*pkd1*, *pkd1b*, *pkd11l*, *pkd112a*, *pkd112b*, *pkd2*, and *pkd21l*) ¹⁷¹ whose function are highly conserved with human orthologs. Several studies have reported Pkd2 expression in developing pronephros (as well as Pkd1) ^{171,172}, ¹⁷³ and in the Kupffer's vesicle (KV) ¹⁷⁴, a transient organ that forms during late gastrulation stages and is required to set left/right asymmetry of the internal organs of the animal ¹⁷⁵. Knockdown of *pkd2* causes pronephric kidney cysts, body axis curvature, organ laterality defects and hydrocephalus, defects that could be rescued by human *PKD2* mRNA expression ¹⁷³. The degree of sequence conservation between zebrafish *pkd2* and human *PKD2* (67% identity) ¹⁷⁶ has led many groups to use zebrafish *pkd2* morphants to study ADPKD.

Furthermore, available genomic data and molecular tools that allow genetic manipulation, such as zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) ¹⁷⁷, have allowed researchers to target genes and generate zebrafish transgenic or mutant lines. These lines can

have fluorescent reporters under the control of tissue-specific promoters, allowing us to observe their expression, and knockout/knockin genes to study their function. In the context of ADPKD, the zebrafish mutant line *curly up (cup)^{tc321}* was created. This line possesses a mutation in *cup (cup^{tc321})*, which encodes the zebrafish ortholog of human *PKD2*, that is likely to be a molecular null based on its non-functional truncated protein¹⁷². This mutant can develop pronephric dilations that never bud off from the tubules, never becoming true cysts, which excludes it as a model for renal ADPKD cystogenesis¹⁷². However, it is useful to study ADPKD extrarenal complications, such as cardiac problems, dorsal axis defects and left-right patterning asymmetry^{172,178}.

Also, zebrafish embryos allow the transient anti-sense knockdown of specific proteins by injecting morpholino oligonucleotides (MOs). MOs are chemically synthesized oligomers, typically of 25 morpholine bases, that are specific via complementary base pairing to the target site of interest to prevent mRNA translation or splicing or to block miRNAs or their targets. Splice-site MOs inhibit zygotic transcripts and closer mimic observed mutations; in contrast, translational blocker MOs can inhibit both maternal and zygotic transcripts, uncovering phenotypes otherwise not observed. The action of the MO is facilitated by its neutrally charged phosphorodiamidate backbone that increases binding affinity for RNA, reduces nonselective interactions and prevents their degradation by nucleases¹⁷⁹.

The use of MOs *versus* mutants is, many times, controversial as MO-induced phenotypes can be often different and more severe than those of the corresponding mutants, which can be due MO's off-target effects¹⁸⁰ or genetic compensation in mutants¹⁸¹. Thus, MOs specificity must be validated by: the use of a second MO, which ideally does not overlap with the first; the use of a mismatch control MO (usually differs in 4-6 nucleotides from tested MO) and the use of mRNA to rescue MO effect, by co-injecting them^{182,183}.

1.9.Objectives

We have seen that cardiovascular problems associated with ADPKD have a great impact in these patients' lives and are a leading cause for morbidity and mortality due to this disease. However, the molecular mechanisms underlying vascular alterations are yet to be understood. Knowing that disruption of PC1 and PC2 function in vascular smooth muscle and endothelium leads to enhanced smooth muscle contractility and impaired endothelial-dependent vasorelaxation¹⁸⁴, which suggests its direct involvement with ADPKD vasculature problems, we wondered if the lack of *Pkd2* would lead to these anomalies.

In order to answer this, we planned to compare *in vivo* vasculature embryonic development, by stereoscopy analysis, in zebrafish in normal conditions and with *Pkd2* loss, which would be achieved in two ways:

- a) Firstly, we would perform *pkd2* knockdown by injecting a translational *pkd2*-MO in embryos of the *Tg(fli1:eGFP; gata1a:dsRed)^{y1;sd2}* zebrafish line. This line expresses GFP under the control of the *fli1* promoter and dsRed under *gata binding protein 1a (gata1a)* promoter, thus tagging vascular endothelial cells and red blood cells, respectively^{185,186}.
- b) Secondly, we would generate a new zebrafish line, *curly up (cup)^{tc321} × Tg(fli1:eGFP; gata1a:dsRed)^{y1;sd2}*, whose *cup^{-/-}* embryos and positive GFP and dsRed signals would further allow us to understand the impact of the lack of *Pkd2* in the vasculature.

The study of *Pkd2* function in zebrafish vasculature could help translate and further understand the function and importance of PC2 in the human vasculature as well as the disrupted pathways which lead to vascular problems in ADPKD patients.

2. MATERIALS AND METHODS

2.1. Zebrafish strains and maintenance

Adult zebrafish lines were maintained in tanks with a controlled aquarium system that provides proper flow and fresh water at 28 °C, with a photoperiod of 13.5 h light and 10 h dark and were fed 3 times a day. The strains used in this study were *Tg(fli1:eGFP, gata1:dsRed)^{y1;sd2}*, *curly-up (cup)^{tc321}* and *curly-up (cup)^{tc321} × Tg(fli1:eGFP, gata1:dsRed)^{y1;sd2}*. The experiments were performed using embryos and larvae obtained from incrosses of the lines. All procedures were approved by the Portuguese Veterinary General Administration (DGAV - Direção Geral de Alimentação e Veterinária) and by the ethics committee of the Nova Medical School/Faculdade de Ciências Médicas, Universidade Nova de Lisboa.

Incrosses were done using 3 to 8 zebrafish couples, according to each line availability. At late afternoon, each couple was placed in a breeding tank with a partition separating male from female. Early in the morning of the next day (at the beginning of the next light cycle) partitions were removed, and eggs collected to a Petri dish after 20 minutes spawning. This ensured egg quality and synchronicity at their one cell-stage development¹⁸⁷. All dead eggs were then removed, and the batch was incubated in E3 embryonic medium (5mM NaCl; 0.17 mM KCl; 0.33 mM CaCl₂; 0.33 mM MgSO₄ and 0.00001% (w/v) methylene blue), at 28 °C until the desired developmental stage. All reagents were purchased from Sigma-Aldrich, USA, except NaCl (VWR International, LLC, USA).

At 24 hpf onwards, embryos were incubated with 200 µM PTU (Sigma-Aldrich, USA) in E3 medium to prevent them from developing pigmentation.

2.2. Generation of the *curly-up (cup)^{tc321} × Tg(fli1:eGFP, gata1:dsRed)^{y1;sd2}* zebrafish line

The *curly-up (cup)^{tc321} × Tg(fli1:eGFP, gata1:dsRed)^{y1;sd2}* zebrafish line was created by crossing *curly-up (cup)^{tc321}* heterozygous females with *Tg(fli1:eGFP, gata1:dsRed)^{y1;sd2}* males. Like described for incrosses, at late afternoon, each couple was placed in a breeding tank without a partition separating male from female. In the morning of the following day, eggs were collected to a Petri dish and incubated in E3 medium. After 24 hpf, embryo surface sanitation, also called embryo bleaching, was performed, by intermittently submerging embryos in 0.004% NaOCl in E3 medium and in E3 medium only, for 5 minutes each, two times.

The embryos were then placed in a new Petri dish (50 embryos per Petri dish) with Pronase (2 mg/mL) (EMD Millipore, MERK, Germany) for 3 minutes at room temperature and incubated at 28 °C. The following day, chorion-free embryos were transferred to a new Petri dish with fresh E3 medium and were screened for GFP and dsRed expression under the *fli1* and *gata1a* promoter, respectively, with the help of a fluorescent stereoscope (Zeiss Lumar V12, Zeiss, Germany).

At 5 days post-fertilization (dpf) all larvae that presented dorsal axis curvature were euthanized with at least 3% NaOCl, after anesthesia in solution of tricaine mesylate (MS-222) (0.16 mg/mL) in E3 medium, as they were *cup^{-/-}* and do not survive longer than 6 dpf. The remaining (*cup^{+/+}* and *cup^{+/-}*) were transferred to an appropriate tank to grow in the same controlled aquarium system as adults and feeding was initiated (3 times a day). After sexual maturity was reached at 3 months post fertilization, incrosses were started with available couples (12 females and over 30 males) to genotype and select heterozygous individuals for *cup^{tc321}*. The offspring of heterozygous couples would include *cup^{+/+}*, *cup^{+/-}* and *cup^{-/-}* at a percentage of 25, 50 and 25%, respectively, and therefore used for our experiments.

2.3. Embryo microinjections

Following instructions of Xin and Duan in “Microinjection of Antisense Morpholinos, CRISPR/Cas9 RNP, and RNA/DNA into Zebrafish Embryos”¹⁸⁸, as well as other works^{189,190}, embryo microinjection was performed using a glass needle that forms a 1 mm capillary tube where the MO is inserted. Once MO reaches the tip, the needle is inserted in the injector (Pneumatic Pico Pump PV820, from World Precision Instruments, USA) and directed into field view of the stereoscope (SMZ745, Nikon Corporation, Japan). With the help of a graticule (S1 Stage Micrometer, 10 mm/0.1 mm, from Pyser Optics, United Kingdom), we proceed to injection volume calibration.

pkd2 knockdown was achieved by injecting one cell-stage embryos with 1.8 ng of translational blocking *pkd2*-augMO (5'-AGGACGAACGCGACTGGAGCTCATC-3')^{172,174}. This MO begins at the start codon and extends through the first exon of the *pkd2* gene. 1.8 ng of a *pkd2*-mismatchMO was used as control (all morpholinos purchased from Gene Tools LLC, USA). From this point forward along this dissertation, *pkd2*-MO and *pkd2*-mismatch-MO injected embryos are named *pkd2*-morphants and mismatch-control, respectively.

2.4. Live fluorescence stereomicroscopy

Live *Tg(fli1:eGFP, gata1:dsRed)^{y1;sd2}* and *curly-up (cup)^{tc321} × Tg(fli1:eGFP, gata1:dsRed)^{y1;sd2}* embryos were dechorionated with clamps, anesthetized with MS-222 (0.16 mg/mL) solution in E3 medium and mounted in 2% (w/v) methyl cellulose (Sigma-Aldrich, USA). Zebrafish lines were observed by fluorescence stereomicroscopy (Zeiss Lumar V12, Zeiss, Germany, software Zen pro 2012). For lateral view of the whole body, magnification was adjusted to each embryo/larvae size, as for dorsal view of the head, zebrafish were observed using 80× magnification.

After observation *curly-up (cup)^{tc321} × Tg(fli1:eGFP, gata1:dsRed)^{y1;sd2}* embryos were gently dismantled by washing them with E3 medium and incubated with 200 μM PTU in E3 medium as mentioned in chapter 2.1., for further observations at later developmental stages.

2.4.1. Dorsal aorta diameter

The diameter of the dorsal aorta, including the caudal aorta, was measured in 5 different locations: at the 5th, 10th, 15th, 20th and 25th myotome (muscle segment), as shown in Figure 2.1., using the ImageJ software (version 1.52p).

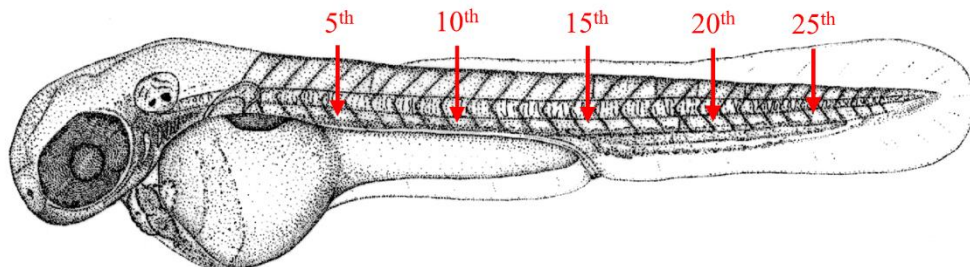


Figure 2.1. – Location of dorsal aorta diameter measurements at the 5th, 10th, 15th, 20th and 25th myotome. (Adapted from¹⁸⁷).

2.5. Widefield and Confocal microscopy

Embryos of 48 hpf *pkd2*-morphants, mismatch-control, non-injected, *cup* control and *cup*^{-/-} mutants (2 of each) were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, USA) during 24 h, maintained at 4 °C. They were then severed and the head was mounted in 2% (w/v) low-melting agarose (Sigma-Aldrich, USA). Intracranial vasculature was observed by microscopy (Zeiss Axio Observer, Zeiss, Germany) with 2x objective. Z-sections were selected as indicated by Zen software, adapting to each embryo's head size. A *pkd2*-morphant was additionally observed by confocal microscopy (Zeiss LSM710, Zeiss, Germany, software Zen Blue 2010b SP1) using a 40× water objective.

2.6. Heart laterality scoring

As previously described¹⁷⁴, the heart jogging was evaluated at 30 hpf by observing the embryos from their ventral side, using a stereoscope (SMZ745, Nikon Corporation, Japan).

2.7. Statistical analysis

Statistical analysis of the obtained results was performed using GraphPad Prism software, version 8.0 (GraphPad Software Inc, USA). Fisher's test was used to test for association between the loss of Pkd2 with cardiovascular anomalies' frequency. Null hypothesis (H_0) conjectures that there is no association between Pkd2 loss and the cardiovascular anomaly tested. When $p < 0.05$, H_0 is rejected, proving association between them. The same test was used to evaluate whether such association was specific for the *pkd2*-MO or, opposing to this, was also present when using the *pkd2*-mismatch-MO.

Dorsal aorta diameters were tested, at each position, for normality with the Shapiro–Wilk test and for significant different variances with the F-test. To test for association with Pkd2 loss and for *pkd2*-MO specificity, differences between samples with a normal distribution were analyzed for statistical significance using:

- a) the Student's *t*-test (two-tailed), if samples' variances were not significantly different;
- b) or the *t*-test with the Welch correction (two-tailed), if samples' variances were significantly different.

Samples whose distribution was significantly different from a normal distribution were analyzed using the Mann–Whitney *U*-test (two-tailed). In every case, differences were considered statistically significant when $p < 0.05$.

3. RESULTS

3.1. Characterization of the *Tg(fli1:eGFP, gata1:dsRed)^{y1;sd2}* zebrafish line

For the first part of this project, it was very important to understand if zebrafish was an adequate model to study the possible effects of *pkd2* loss on the vasculature. Our group has previously shown the KV, an organ transiently present in early embryonic life of the fish to establish internal body laterality^{175,191}, to be a good model system to study the molecular mechanisms involved in the process of kidney cyst inflation in ADPKD^{174,192}. Indeed, the knockdown of *pkd2* lead to an increase in KV volume, through the stimulation of CFTR^{174,192}.

Therefore, we wondered if zebrafish could also be used as a model to study the vascular abnormalities associated with ADPKD. Many groups have previously exploited the potential of zebrafish lines to study not only vascular development *in vivo*^{165,193,194}, but also vascular disease^{195,196}. Moreover, Walcott and Peterson suggest embryonic zebrafish as an ideal organism for the study of intracranial aneurysms, which, as we know, is one of the main vascular complications associated with ADPKD¹⁹⁶.

Taking this into consideration, we first thought of using *Tg(fli1:eGFP)^{y1}* zebrafish line to study these vasculature alterations *in vivo* throughout embryonic development. This transgenic line expresses GFP under the control of the promoter of the gene *fli1* *proto-oncogene, ETS transcription factor*¹⁹⁷, also known as *Friend leukemia integration 1 transcription factor*¹⁹⁸. *Fli1* is initially expressed in the posterior lateral mesoderm, therefore, this line labels vascular endothelial cells throughout embryogenesis¹⁸⁶. It can also be found in the mesenchyme of aortic arches and the developing cartilage of the jaw¹⁹⁷. In our work, the GFP expression was detected in embryos at 48 hpf, through live-fluorescence stereomicroscopy (Zeiss Lumar V12).

These first results, coincidental with previous works^{199,200}, allowed us to observe major trunk vessels at this stage, such as the dorsal aorta (DA), which extends all the way through the yolk until the beginning of the tail, the caudal artery (CA), the intersegmental vessels (Se), that separate each myotome, and the dorsal longitudinal anastomotic vessel (DLAV) (Figure 3.1 – A). At the ventral portion of the embryo, we also observe the posterior cardinal and caudal vein (PVC and CV).

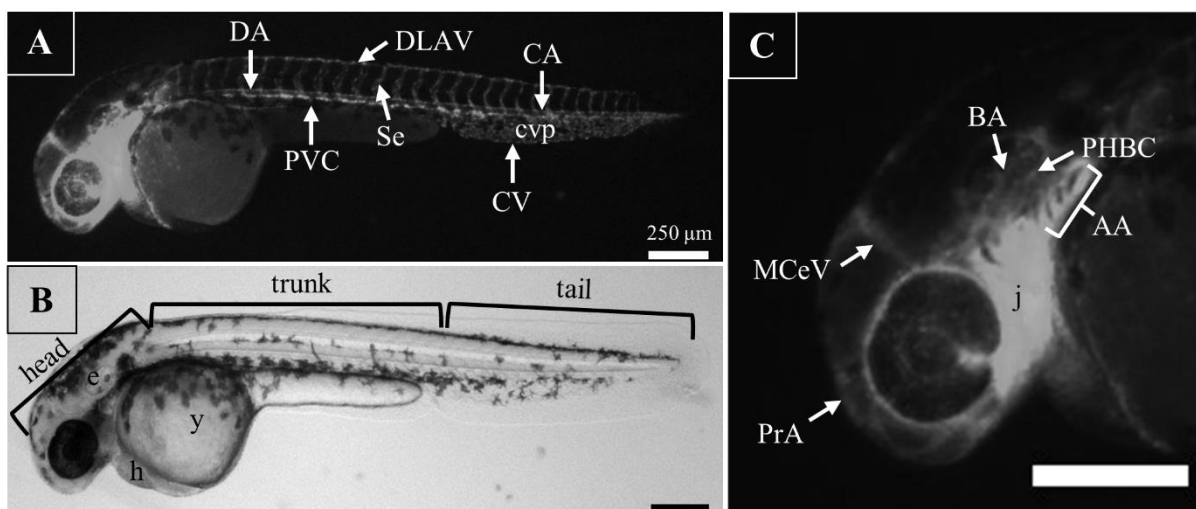


Figure 3.1. – Characterization of *Tg(fli1: eGFP)^{y1}* zebrafish line. Left lateral view of an embryo at 48 hpf acquired by fluorescence (A and C) and brightfield stereomicroscopy (B) (Zeiss Lumar V12). Expression of GFP, tagging vascular

endothelial cells, in the A) trunk and C) head. B) At this developmental stage it is already visible the skin pigmentation. Scale bars: 250 μ m. Structures and vessels represented: basilar artery (BA), aortic arches (AA), caudal artery (CA), caudal vein (CV), caudal vein plexus (cvp), dorsal aorta (DA), dorsal longitudinal anastomotic vessel (DLAV), ear (e), forming jaw (j), heart (h), intersegmental vessels (Se), middle cerebral vein (MCeV), primordial hindbrain channel (PHBC), prosencephalic artery (PrA).

Likewise, the caudal vein plexus (cvp) presents a high signal of GFP. This plexus consists of post-vent vessels that form an interconnecting venous vascular network, formed during a very active period of angiogenic sprouting, which begins at 25 hpf²⁰¹, having therefore the potential of being a good readout for vascular endothelial anomalies. GFP is also detected in the heart to some extent, as it was possible to observe a faint GFP signal.

It was also very noticeable that the most marked structures in the embryo with GFP were the mesenchyme of the aortic arches (AA), as well as the vessels themselves, and the mesenchyme of the forming jaw (j) (Figure 3.1. – C), which is consistent with previous reports of Fli1 expression in derivatives of the cranial neural crest. This intense GFP expression affected, however, our ability to accurately distinguish the main vessels in the head, at least by using the Zeiss Lumar V12 stereoscope. As such, the only ones that were clearly perceptible from this lateral view, consisted of the basilar artery (BA), prosencephalic artery (PrA), middle cerebral vein (MCeV) and primordial hindbrain channel (PHBC) (Figure 3.1. – C).

Although this zebrafish line seemed appropriate, we quickly realized that it was lacking in some respects and didn't give us all the insight we were looking for. Especially if further on there was a need for us to be able to discriminate anomalies in the vasculature related to ADPKD, that could cause alterations of the blood flow or internal hemorrhages.

As such, we decided to use the *Tg(fli1:eGFP; gata1a:DsRed)^{y1;sd2}* zebrafish line (Figure 3.2). Like *Tg(fli1:eGFP)^{y1}*, this line expresses GFP under the control of the *fli1* promoter. But, as a double transgenic, it additionally expresses dsRed under *gata binding protein 1a* (*gata1a*) promoter, a transcription factor that regulates erythropoiesis, thus tagging red blood cells¹⁸⁵.

From the analyses of first obtained images, we encountered two drawbacks. First, as we were mounting the embryos in 2% methylcellulose and as embryos have different widths throughout their length, it was not always possible to obtain a clear image of all vessels in the same focal plan in the lateral view. Therefore, we aimed to obtain several images per embryo, when such difficulty was presented, which increased the number of experiments. Secondly, we also noticed that embryos' pigmentation, already observed in the *Tg(fli1:eGFP)^{y1}* zebrafish line (Figure 3.1. – B), was many times affecting the quality of our images. Therefore, we decided to treat *Tg(fli1:eGFP; gata1a:dsRed)^{y1;sd2}* embryos from 24 hpf onwards with PTU to prevent them from developing pigmentation (Figure 3.2 – A).

Expression of GFP was detected in the same structures and vessels that were mentioned before (Figure 3.1.) in the trunk, which includes dorsal aorta (DA), intersegmental vessels (Se), dorsal longitudinal anastomotic vessel (DLAV), posterior cardinal vein (PCV), caudal vein (CV) and caudal vein plexus (cvp) (Figure 3.2. – B). As expected, the pre-treatment of the embryo with PTU proved to be very effective and allowed to disclose the expression of GFP on the yolk common cardinal vein (CCV), the mesenchyme of the developing fin bud (Figure 3.3. – B). This time, in order to obtain a better image of the intracranial vascular structure, we additionally captured dorsal images of the embryo's head (Figure 3.3 – C). The PTU pre-treatment also gave us more detail in the intracranial vasculature.

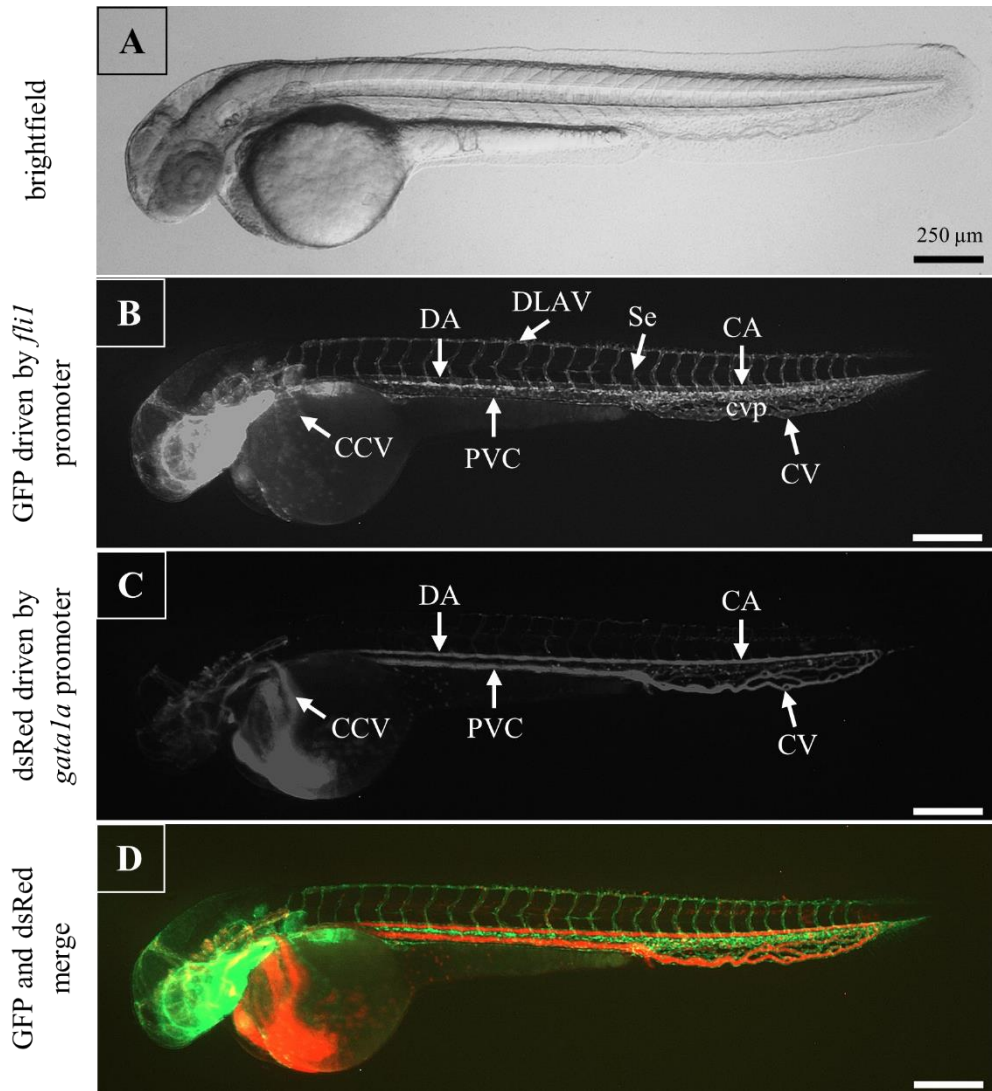


Figure 3.2. – Characterization of *Tg(fli1:eGFP; gata1a:dsRed)^{y1;sd2}* zebrafish line. Left lateral view of embryo at 48 hpf acquired by brightfield (A) and fluorescence stereomicroscopy (B – D) (Zeiss Lumar V12). A) Treatment with PTU prevented pigmentation. B) Expression of GFP under the control of the *fli1* promoter, tagging vascular endothelial cells. C) Expression of dsRed under the control of *gata1a* promoter, tagging red blood cells. D) Merge of the expression of both GFP (in green) and dsRed (in red). Scale bars: 250 μ m. Structures and vessels represented: caudal artery (CA), caudal vein (CV), caudal vein plexus (cvp), common cardinal vein (CCV), dorsal aorta (DA), dorsal longitudinal anastomotic vessel (DLAV), intersegmental vessels (Se).

This zebrafish line showed to be extremely advantageous, as the observation of dsRed expression driven by *gata1a* promoter enabled us to get a better view of vessels, that due to the high expression of GFP driven by *fli1* promoter in the entire vasculature, as well as other mesenchymal structures, remained imperceptible. In fact, the *in vivo* observation of blood circulation, revealed as expected that from the heart to the trunk, blood flows mainly through vessels with higher diameter such as the dorsal aorta (DA) and caudal aorta (CA), returning via caudal vein (CV) (sometimes diverging through some vessels in the caudal vein plexus) and consecutively posterior cardinal vein (PCV), from which blood flows back through the common cardinal veins (CCV) into the heart. Additionally, erythrocytes could also be seen in vessels with smaller diameter such as the dorsal longitudinal anastomotic vessel (DLAV) and intersegmental vessels (Se) (Figure 3.2. – C).

When looking at the embryos' head from a dorsal point of view, we noticed that the intracranial vascular network is a complex primitive plexus, with many circulatory loops, at 48 hpf. Comparing it with that described in Isogai and colleagues' study ²⁰¹, through *fli1* driven GFP levels together with *gata1a* driven dsRed, we were able to understand that at this stage, regardless of variance between embryos morphology, the main distinguishable vessels comprised: more superficially, the mesencephalic vein (MsV), middle cerebral vein (MCeV) and dorsal middle junction (DMJ); followed by the basal communicating artery (BCA), posterior communicating segment (PCS), basilar artery (BA), primordial hindbrain channel (PHBC), lateral dorsal aorta (LDA) and sometimes the metencephalic artery (MtA) and cranial division of the internal carotid artery (CrDI).

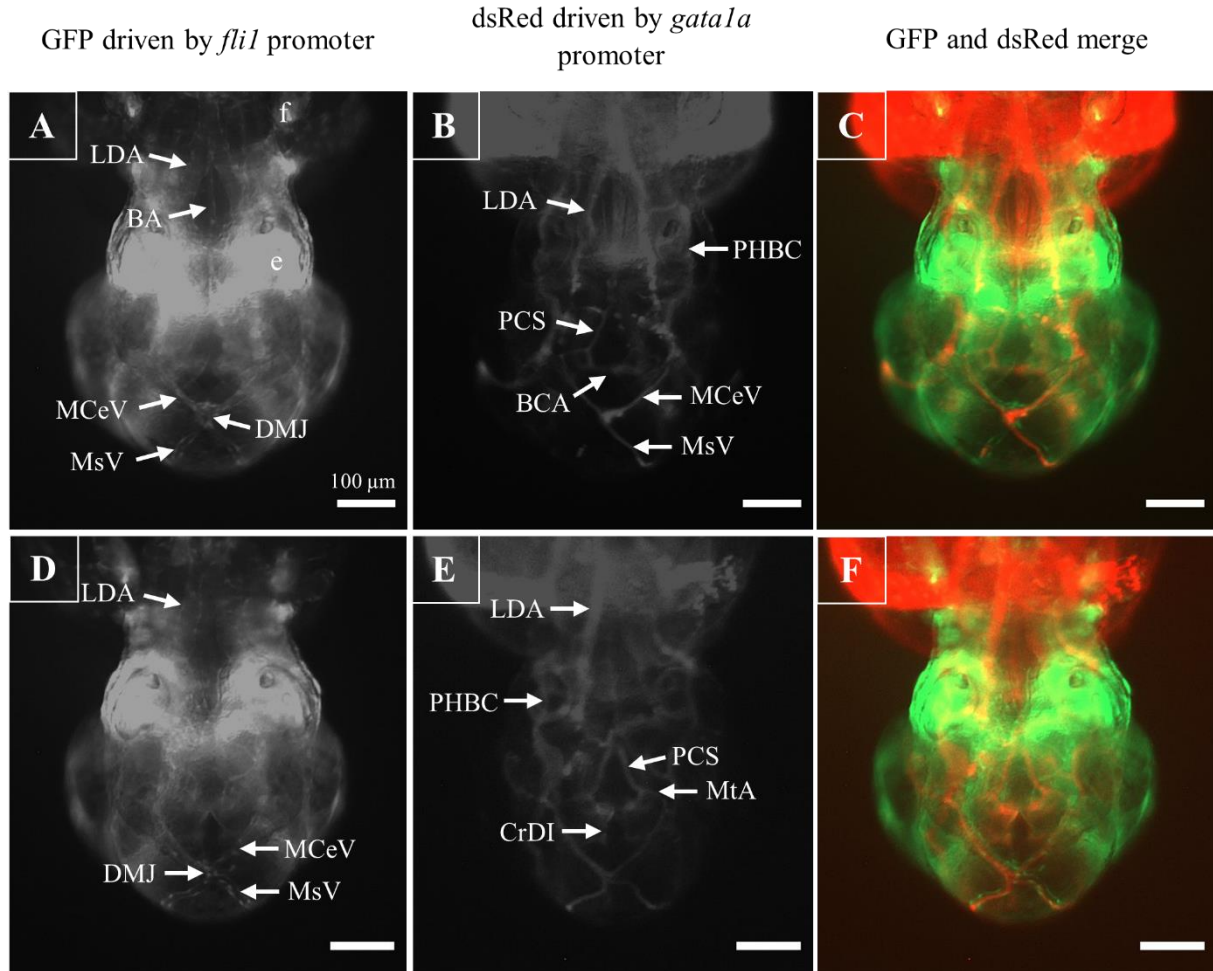


Figure 3.3. – Characterization of the *Tg(fli1:eGFP; gata1a:dsRed)^{y1;sd2}* zebrafish line. Dorsal view of embryo head at 48 hpf acquired by fluorescence stereomicroscopy (Zeiss Lumar V12). A), D) expression of GFP driven by *fli1* promoter. B), E) expression of dsRed driven by *gata1a* promoter. C), F) merge of both GFP and dsRed. Scale bars: 100 μ m. Structures and vessels represented are: basal communicating artery (BCA), basilar artery (BA), cranial division of the internal carotid artery (CrDI), dorsal middle junction (DMJ), ear (e), fin bud (f), lateral dorsal aorta (LDA), middle cerebral vein (MCeV), mesencephalic vein (MsV), metencephalic artery (MtA), posterior communicating segment (PCS), primordial hindbrain channel (PHBC).

To conclude the characterization of this zebrafish line, it is important to refer that the intensity of the GFP and dsRed signals were variable among the embryos and that the yolk of zebrafish embryos and larvae is naturally auto-fluorescent, features that diffculted our analyses. Indeed, at 24 hpf dsRed signal was not strong and was difficult to detect, preventing observations at this stage of development.

However, at 48 hpf dsRed expression was easily detected and provided a clear observation of erythrocytes.

3.2. Impact of *pkd2* knockdown

By live analysis of the *Tg(fli1:eGFP; gata1a:dsRed)^{y1;sd2}* zebrafish embryos, we then proceeded to study if *pkd2* knockdown has an influence in vascular formation and structure that could underlie the well-known ADPKD vascular problems³². To guarantee morpholino efficiency, which is limited by dilution, since differential mitotic activity in different cellular lineages leads to asymmetric distribution, and degradation^{179,202}, the embryos injected with *pkd2*-MO were observed no longer than 48 hpf.

3.2.1. Characterization of zebrafish defects

The most common and penetrant phenotype of *pkd2* knockdown in zebrafish embryos, that has been widely reported before^{172,203}, is the dorsal axis curvature (curly up) (Figure 3.4. – C). Mangos and his team showed evidence that both *pkd1a/b* and *pkd2* regulate collagen mRNA and protein expression and that the overexpression of this structural protein of the extracellular matrix (ECM) could be responsible for this defect²⁰³. Therefore, we used this phenotype as a guarantee of the success of *pkd2*-knockdown among our pools of injected embryos, being only those selected for analysis.

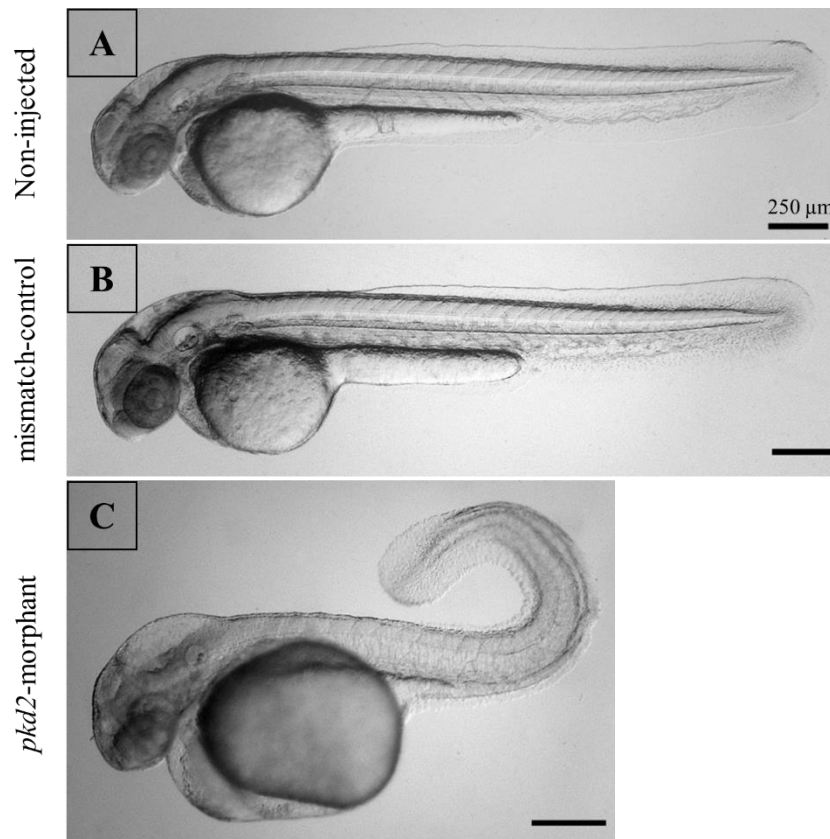


Figure 3.4. – Dorsal axis curvature (curly-up) in *pkd2*-morphant. Left lateral view of embryo at 48 hpf acquired by brightfield stereomicroscopy (Zeiss Lumar V12). A) non-injected embryo. B) mismatch-control. C) *pkd2*-morphant. Scale bars: 250 μm.

To begin with, a singular defect that caught our attention, that was present in some of the *pkd2*-morphants (12 %), was the malformation of the caudal vein plexus, which lead to an accumulation of erythrocytes (Figure 3.5. – D). As mentioned before, this network is formed through angiogenesis, a process that is very active at our window of observation, therefore, besides ECM alterations, *pkd2*-knockdown could have a role in this process. Supporting this evidence, a few *pkd2*-morphants (24 %) had intersegmental vessels incompletely formed (Figure 3.5. – E, F), which are among the first angiogenic vessels to form in all vertebrates²⁰⁴. However, these defects were not significantly associated with Pkd2 loss, which was made obvious when comparing mismatch-control with non-injected embryos ($p=0,0418$), suggesting an off-target effect or possibly a slight delay in development associated with morpholino use.

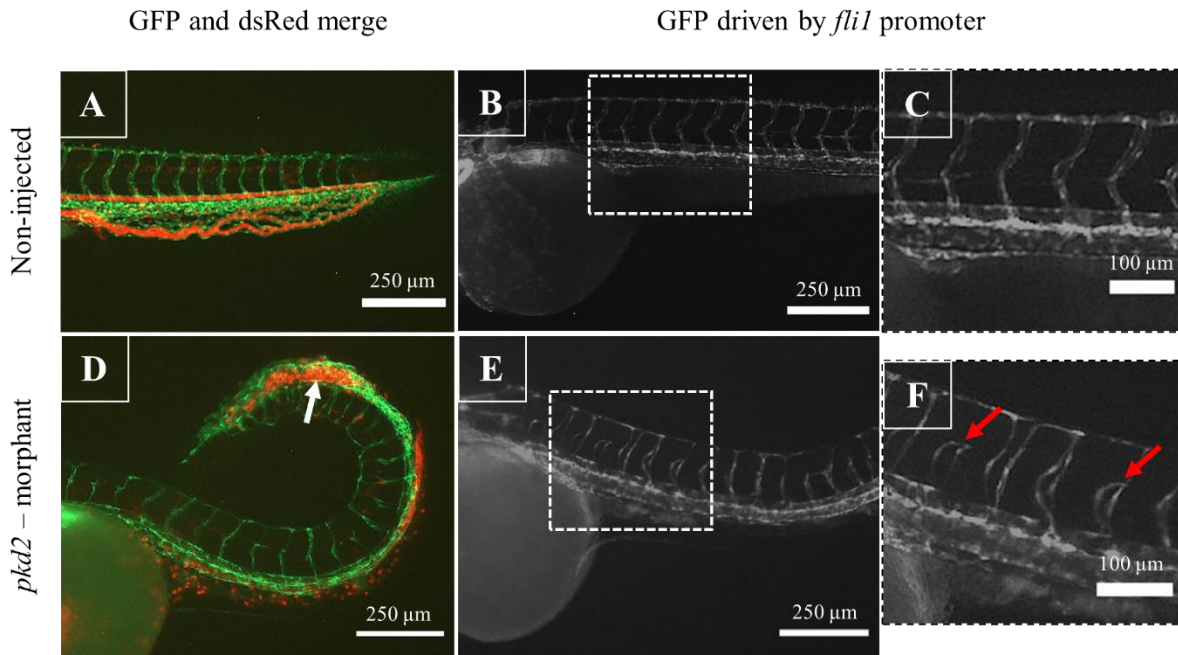


Figure 3.5. – Malformation of the caudal vein plexus and incomplete formation of intersegmental vessels in *pkd2*-morphants. Left lateral view of non-injected (A, B, C) and *pkd2*-morphants' (D, E, F) tail vessels at 48 hpf acquired by fluorescence stereomicroscopy (Zeiss Lumar V12). A), D) merge of GFP expression driven by *fli1* promoter and dsRed expression driven by *gata1a* promoter. B), C), E), F) expression of GFP driven by *fli1* promoter. White arrows: malformation of caudal vein plexus. Red arrows: intersegmental vessels incompletely formed. Scale bars: 250 μm (A, B, D, E) and 100 μm (C, F).

Another feature that becomes more common in zebrafish embryos with *pkd2* knockdown is heart *situs* defects, due to left-right asymmetry, which can result in a mirror arrangement of the organs (*situs inversus*) or other abnormal organ position combinations (heterotaxia) instead of the correct arrangement (*situs solitus*)²⁰⁵.

We observed some *pkd2*-morphants with heart laterality defects (16%), which was significantly associated with lack of Pkd2 ($p=0.0123$), given that no association was found between mismatch-control and non-injected embryos. However, the percentage of occurrence was lower than what has been previously observed by our group (32%)²⁰⁵, suggesting the need for a higher number of embryo observations. Moreover, this defect is related to Pkd2 function in the KV and not on the vasculature, therefore, we focused on other cardiac anomalies.

Cardiac edema can be provoked by environmental stress as well as genetic changes, particularly during a critical period in heart development, which can culminate in heart failure and circulatory collapse ²⁰⁶. In our observations this defect was reported in 56% of *pkd2*-morphants, showing a statistically significant association ($p < 0.0001$) between the knockdown of *pkd2* and cardiac edema. Moreover, no significant association to the use of the *pkd2*-mismatch-MO was found, assuring that cardiac edema was not a toxic effect of morpholinos. Indeed, cardiac edema was not detected in any embryo of the control groups (non-injected and mismatch-MO injected embryos) (Figure 3.6. – black arrow C, D). Furthermore, blood seemed to concentrate around the heart and blood flow seemed impaired all throughout the body, as suggested by an accumulation of dsRed tagged erythrocytes, being much slower than normal (data not shown).

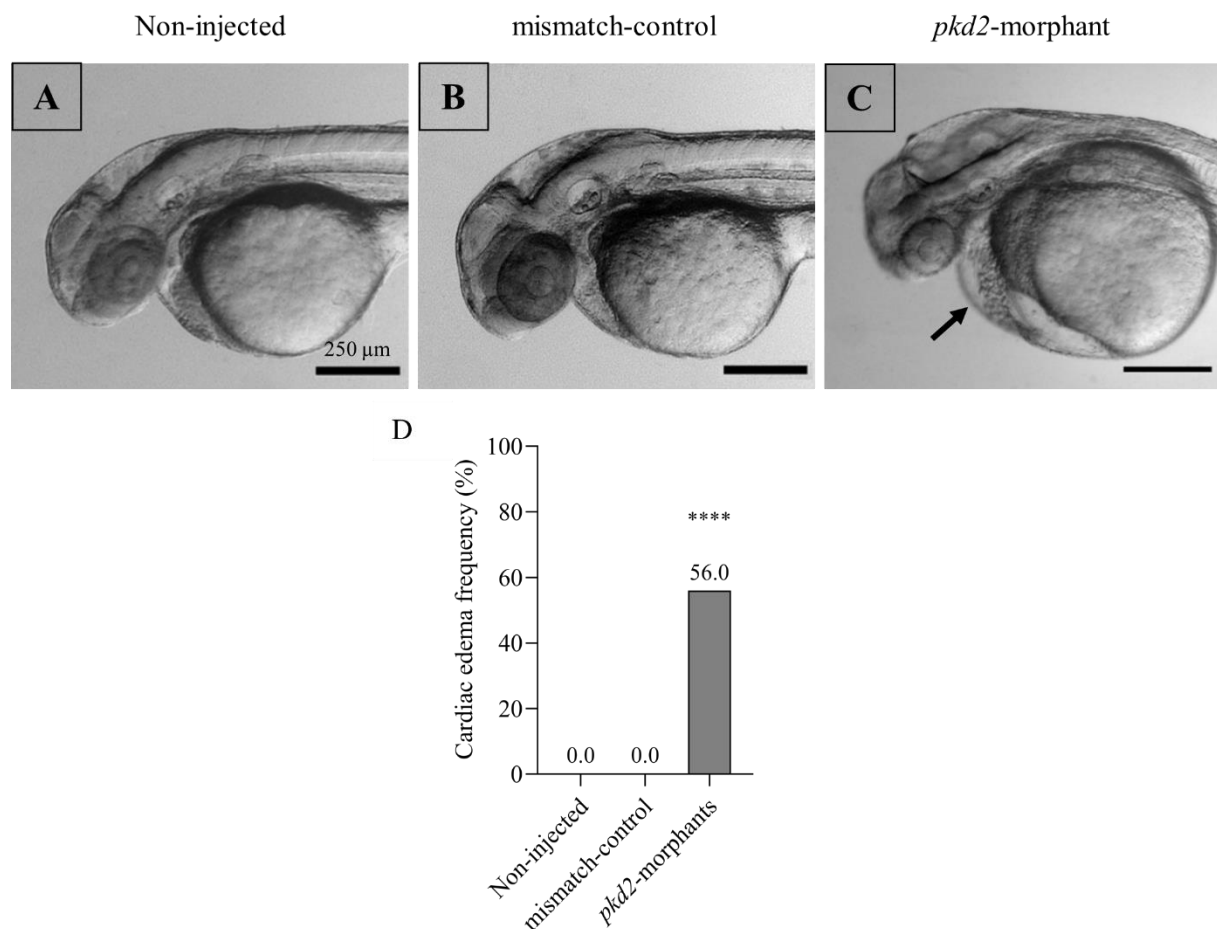


Figure 3.6. – Cardiac edema in *pkd2*-morphants. Left lateral view of a non-injected (A), mismatch-control (B) and *pkd2*-morphant (C) head at 48 hpf acquired by brightfield stereomicroscopy (Zeiss Lumar V12). Black arrow: cardiac edema. Scale bar: 250µm. D) Frequency of occurrence of cardiac edemas in non-injected (n=47), mismatch-control (n=26) and *pkd2*-morphants (n=25). **** $p < 0.0001$, meaning a significant association between the specific use of the *pkd2*-MO (i.e., when compared to the non-injected control) and cardiac edema, by Fisher's-test.

By observing the head of *pkd2*-morphants from a dorsal point of view, it was clear that dsRed tagged erythrocyte circulation was impaired in most embryos, suggesting an impairment of blood circulation. This anomaly was characterized by either no circulation detection or very slow blood flow. In the latter it seemed that it was particular to vessels such as: lateral dorsal aorta (LDA), basal communicating artery (BCA), posterior communicating segment (PCS), middle cerebral vein (MCeV), metencephalic artery (MtA). It was also observed in the vasculature of the eye, that at this stage is composed of primordial hindbrain channel (PHBC) and cranial division of the internal carotid artery (CrDI) vessels ²⁰⁷ (Figure 3.7). In embryos close to no circulation in the head, erythrocytes were only detected in lateral dorsal aorta and middle cerebral vein, probably due to their larger diameter. However, PHBC never showed blood circulation in any of the *pkd2*-morphants with blood circulation impairment.

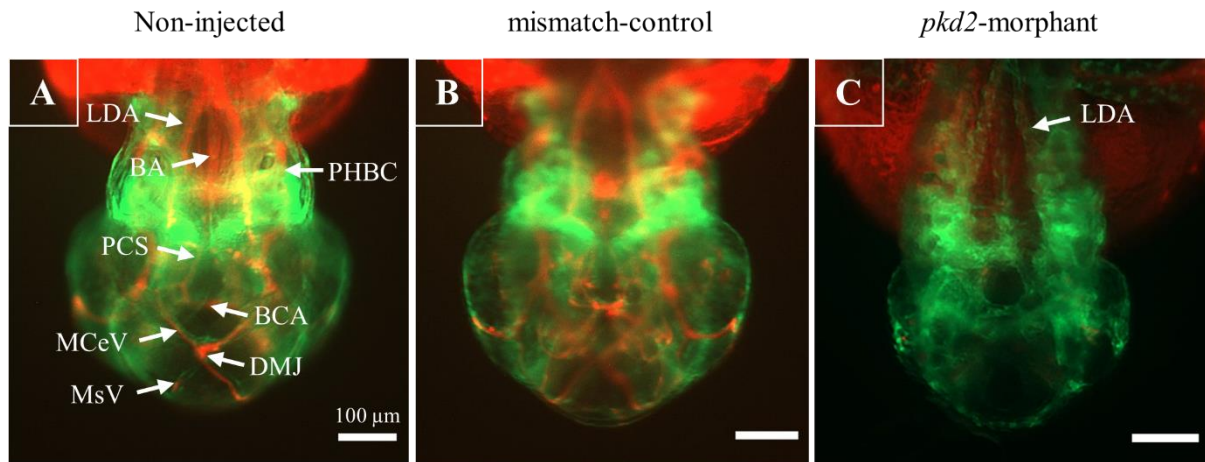


Figure 3.7. – Impaired circulation of erythrocytes in *pkd2*-morphant's head blood vessels. Dorsal view of merge GFP driven by *fli1* promoter and dsRed by *gata1a* promoter expression of a non-injected (A), mismatch-control (B) and *pkd2*-morphant (C) head at 48 hpf acquired by fluorescence stereomicroscopy (Zeiss Lumar V12). Scale bars: 100 μm.

Moreover, a characteristic that show cased vascular problems was the apparent presence of hemorrhages in the head (Figure 3.8. - C). Indeed 31.3% of *pkd2*-morphants had hemorrhages, compared to the 15.4% of mismatch-control and 10.6% of non-injected embryos. By applying the Fisher's test, we found that there is a statistically significant association between the knockdown of Pkd2 and the presence of intracranial hemorrhages ($p=0.0385$). And, after comparing mismatch-control and non-injected embryos, no association was found between the use of *pkd2*-mismatch-MO and hemorrhages ($p=0.7124$), refuting morpholino toxicity.

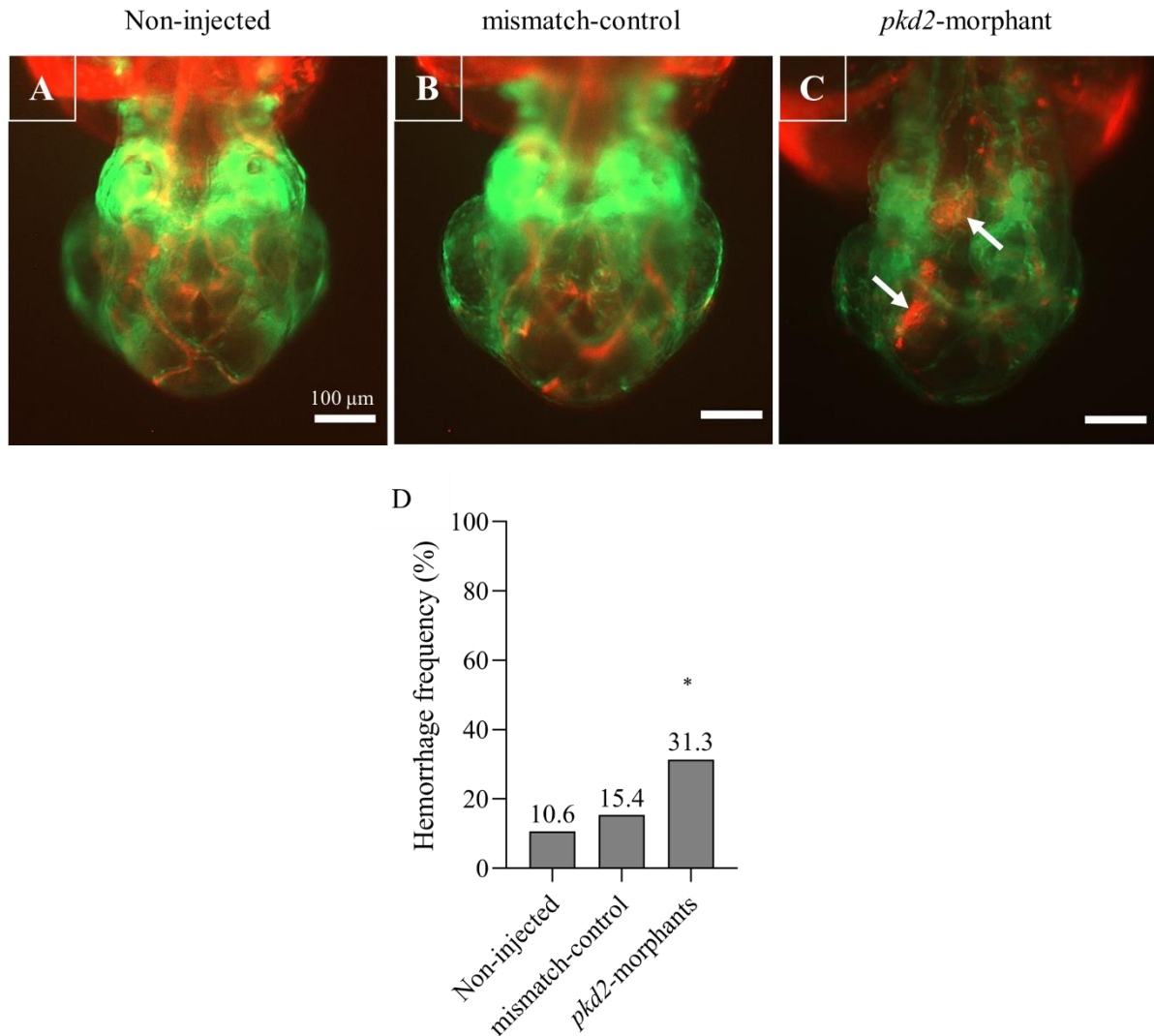


Figure 3.8. – Intracranial hemorrhage in *pkd2*-morphants. Dorsal view of merge GFP driven by *flil* promoter and dsRed by *gata1a* promoter expression of a non-injected (A), mismatch-control (B) and *pkd2*-morphants' (C) head at 48 hpf acquired by fluorescence stereomicroscopy (Zeiss Lumar V12). White arrows: hemorrhages. Scale bars: 100 μ m. D) Frequency of occurrence of hemorrhages in non-injected (n=47), mismatch-control (n=26) and *pkd2*-morphants (n=32). * $p < 0.05$, meaning a significant association between the specific use of the *pkd2*-MO (*i.e.*, when compared to the non-injected control) and hemorrhage, by Fisher's-test.

Finally, hydrocephalus was the other main defect noticed in *pkd2* knockdown (Figure 3.9. – D). This condition involves a significant buildup of fluid in the ventricles of the brain, that can be caused due to a problem with the mechanisms that enable blood vessels to absorb this fluid²⁰⁸. Therefore, it can also give us a readout on vascular anomalies. Correspondingly, 68% of *pkd2*-morphants had hydrocephalus whereas the control groups were mostly unaffected. Again, a statistically significant association between the knockdown of *pkd2* ($p < 0.0001$), which was specific for the use of *pkd2*-MO ($p < 0.0001$), and hydrocephalus was found (Figure 3.9. – D). Additionally, *pkd2*-morphants heads appeared to be smaller than those of non-injected and mismatch-control siblings, which might hint to the involvement of Pkd2 in the development of these tissues as well.

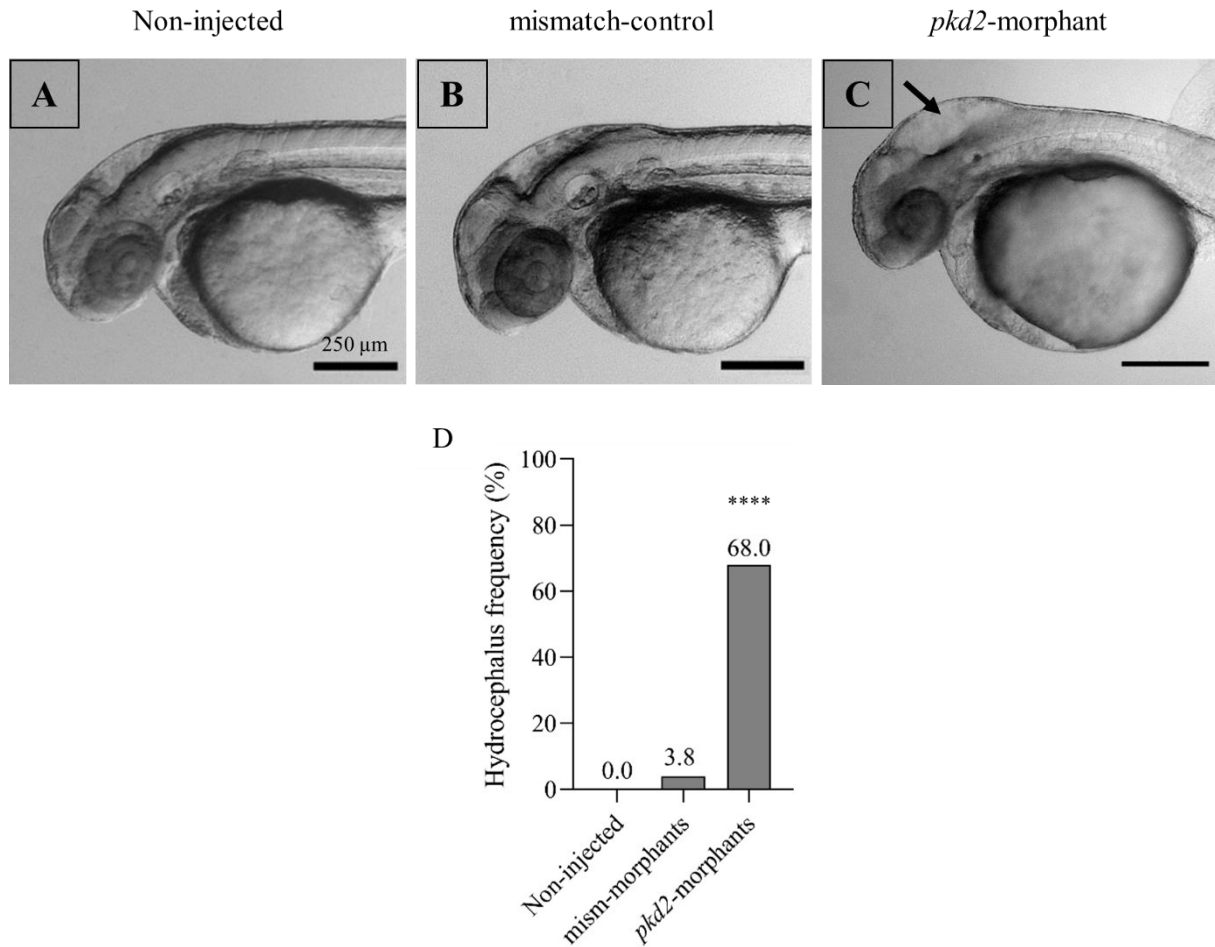


Figure 3.9. – Hydrocephalus in *pkd2*-morphants. Left lateral view of a non-injected (A), mismatch-control (B) and *pkd2*-morphant (C) head at 48 hpf acquired by brightfield stereomicroscopy (Zeiss Lumar V12). Black arrow: hydrocephalus. Scale bars: 250µm. D) Frequency of occurrence of hydrocephalus in non-injected (n=47), mismatch-control (n=26) and *pkd2*-morphants (n=32). **** $p < 0.0001$, meaning a significant association between the specific use of the *pkd2*-MO (i.e., when compared to the non-injected control) and hydrocephalus, by Fisher's-test.

In review, from our analysis we found that *pkd2* knockdown was associated with the following cardiovascular alterations: heart situs defects, cardiac edema, hemorrhages and hydrocephalus.

3.2.2. Dorsal aorta diameter

To quantify vascular alterations that could arise in *pkd2* knockdown, we decided it was important to measure the width of a vessel that could be easily observable through Zeiss Lumar V12 stereoscope. As reported by others, Pkd2 is expressed in endothelial cells and vascular smooth muscle cells^{54,84}, which provide collagen synthesis and maintenance of a healthy extracellular matrix¹⁴⁵. In ADPKD, polycystins haploinsufficiency changes the vasculature and dysregulates pathways that lead to hypertension, extracellular matrix changes and inflammation¹³⁹. Therefore, dorsal aorta showed great potential since it is the main artery along the trunk, reaches its maximum embryogenic caliber at approximately 48 hpf²⁰⁹ and could be affected by changes in vasculature, blood pressure and blood flow. Indeed, ADPKD patients have shown higher rates of aortic defects (aneurysms and dissection) than the general population²¹⁰.

We proceeded to measure the diameter of the dorsal aorta, including the caudal aorta, in 5 different locations: at the 5th, 10th, 15th, 20th and 25th myotome, using the ImageJ software. These measurements started at the 5th myotome because the first segments of dorsal aorta are many times hidden by the yolk. Analysis of *pkd2*-morphants, showed that, throughout this vessel, diameter was always significantly narrower than non-injected ($p < 0.0001$). Furthermore, dorsal aorta diameter of mismatch-control never differed from non-injected embryos' (Table 3.1. and Figure 3.10.).

Table 3.1. – Mean and standard deviation (SD) of dorsal aorta diameter measurements at the 5th, 10th, 15th, 20th and 25th myotome and number (n) of analyzed non-injected, mismatch-control and *pkd2*-morphants' embryos.

		5 th	10 th	15 th	20 th	25 th
Non-injected	Mean (μm)	24.051	24.903	24.057	21.911	18.761
	SD (μm)	2.608	2.522	2.944	2.279	2.020
	n	41	41	41	41	41
mismatch-control	Mean (μm)	23.824	24.485	24.068	22.523	19.869
	SD (μm)	2.657	2.227	2.638	2.269	2.110
	n	26	26	26	25	25
<i>pkd2</i> -morphants	Mean (μm)	17.753	17.758	13.474	11.235	9.162
	SD (μm)	3.640	4.310	4.998	2.743	2.972
	n	24	22	21	18	11

The reduction in number of measurements taken throughout *pkd2*-morphants' dorsal aorta (Table 3.1.) was due to the difficulty in the clear observation of the endothelium of the latest segments of dorsal aorta amidst the abnormalities observed in these embryos, as well as the difficulty intense curly-up presented in focusing and capturing this vessel at all measurement locations.

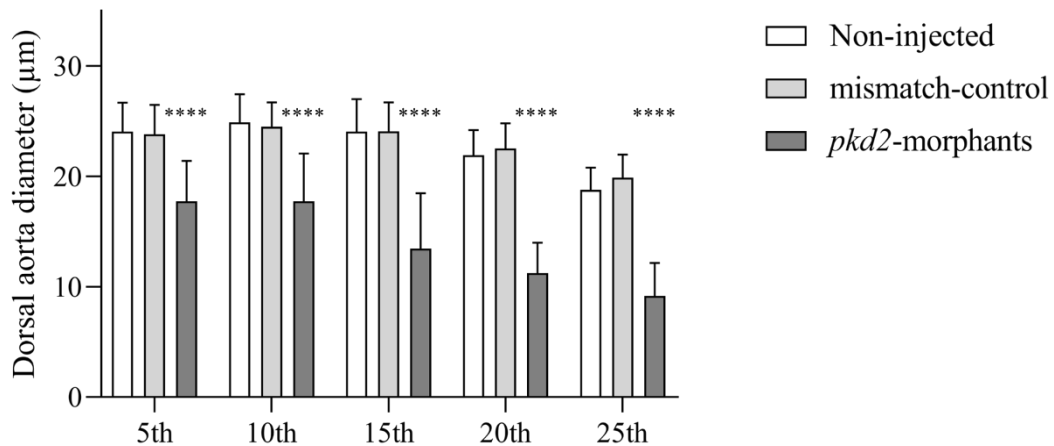


Figure 3.10. – Dorsal aorta diameter (μm) of 48 hpf *pkd2* morphants at the 5th, 10th, 15th, 20th and 25th myotome. **** $p < 0.0001$, significantly different from respective non-injected embryos by Mann-Whitney *U* test, Student's *t*-test and *t*-test with the Welch correction. *pkd2*-morphants' 5th and 25th, and non-injected 10th measurements do not follow a normal distribution; remaining measurements follow a normal distribution with equal variances except for *pkd2*-morphants' and non-injected 15th measurements. As such, Mann-Whitney *U* test was used to compare 5th, 10th and 25th measurements between *pkd2*-morphants and non-injected and 10th measurements between mismatch-control and non-injected; *t*-test with the Welch correction was used to compare 15th measurements between *pkd2*-morphants and non-injected; Student's *t*-test was used to compare all remaining measurements between *pkd2*-morphants and non-injected and mismatch-control and non-injected.

Taking into consideration previous studies that showed enhanced vascular smooth muscle contractility due to polycystins impairment and the role of *Pkd2* in the vessels, these results were similar to what was expected and showed a significant association between *pkd2* knockdown and the reduction of the dorsal aorta caliber.

3.3.Characterization of the *curly-up* (*cup*)^{tc321} × *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2} mutant zebrafish line

For the second part of this work, we generated a new zebrafish line *curly-up* (*cup*)^{tc321} × *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2} by crossing heterozygous individuals of the mutant line *curly up* (*cup*)^{tc321} with individuals of the *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2} line. The former possesses a mutation in *cup*, which encodes the zebrafish ortholog of human *PKD2*, that is likely to be a molecular null based on its non-functional truncated protein¹⁷². The *curly-up* (*cup*)^{tc321} × *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2} mutant zebrafish line would offer us the opportunity to observe the embryos after 48 hpf and at least until 6 dpf, as *cup*^{-/-} don't survive past this time due to defects and malformations.

After the line was 12 weeks old, age at which zebrafish are developed enough to start crossings, the couples (*cup*^{+/+} and *cup*^{+/-}) were screened for the presence of the (*cup*)^{tc321} mutation since only heterozygous couples' offspring would include *cup*^{-/-} (25%), *cup*^{+/-} (50%) and *cup*^{+/+} (25%) embryos.

To characterize this line, we used embryos that did not present the dorsal axis curvature, considering them our *cup* control group. These included *cup*^{+/+} embryos as well as *cup*^{+/-} as there was no distinguishable trait between them. This characterization was done in the exact same way as the previous line, however 48 hpf embryos' development was further followed at 72 and 96 hpf. At each of these stages we registered which were the main morphologic changes in order to understand what characteristics were typical of normal cardiovascular development. From our analysis, we found that the main distinction from 48 to 96 hpf was the increase in both length and number of vessels in the head, i.e. the increase of the complexity of the intracranial vasculature, which has been reported before¹⁹³, and the development of important vessels of the digestive system.

Describing these results in more depth, and making a brief comparison to 48 hpf *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2} non-injected embryos, 48 hpf *cup* control embryos seemed to develop slightly faster and were a little more sturdy (wider and shorter). Indeed, following Kimmel's description of the head-trunk angle as a function of hours of development¹⁸⁷, *cup* control at 48 hpf showed a head-trunk angle characteristic of a slightly more advanced developmental stage. Nevertheless, both lines were very alike in trunk vasculature, and GFP and dsRed expression were detected in all structures and vessels reported for the *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2} line (Figure 3.11. – A, D, G). The same was verified for the intracranial vasculature (Figure 3.12. – A, B, C) and blood flow (data not shown), which were equivalent to *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2} non-injected embryos.

Following these embryos at 72 hpf, besides the already mentioned vessels, we were able to observe the hepatic portal vein (HPV), a vessel formed during the last stages of liver budding morphogenesis²¹¹, as well as the suprainstestinal artery (SIA) and subintestinal vein (SIV), which reflects digestive system development (Figure 3.11. – B, E) Progress is also evident in the simplification of the caudal vein plexus, smaller yolk, straighter head as well as the protrusion of the mouth (Figure 3.11. – H).

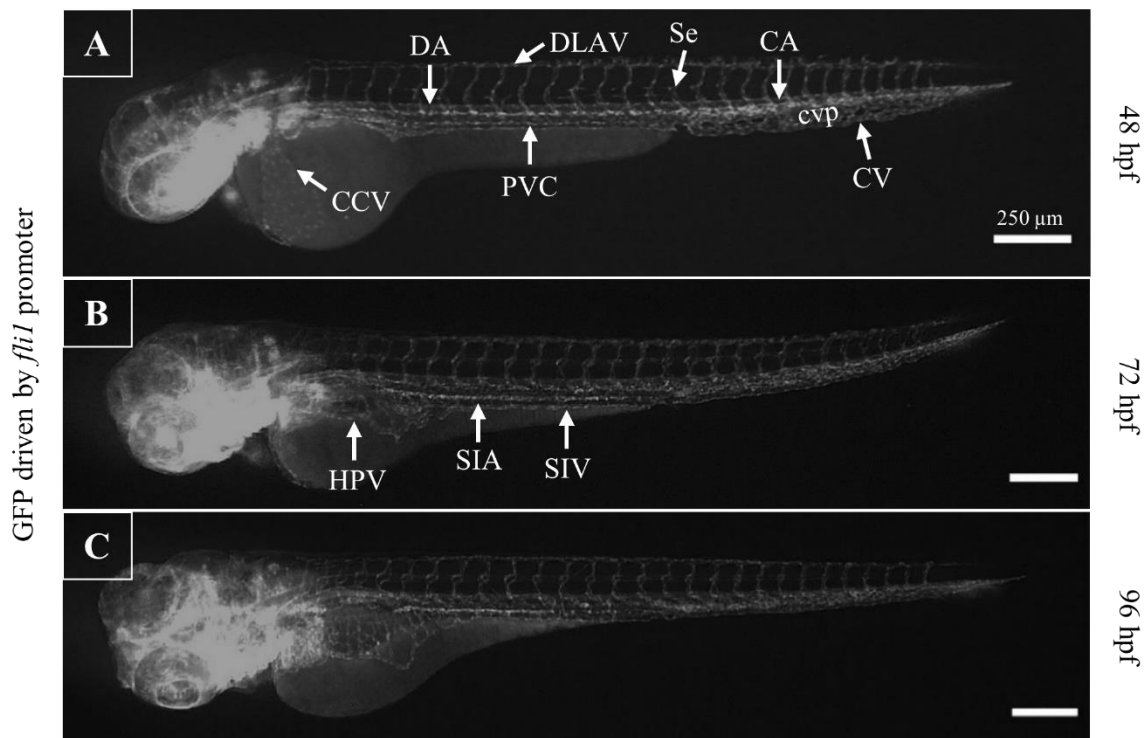
As for the intracranial vasculature, we noticed a significant increase in the length and number of vessels, instead of the primitive plexus with many circulatory loops at 48 hpf. Due to its growing

number and because the stereoscope (Zeiss Lumar V12) doesn't have enough resolution, many of the vessels remained undistinguishable. Even so, we were able to identify the following: basilar artery (BA), posterior communicating segment (PCS), lateral dorsal aorta (LDA), primordial midbrain channel (PMBC), anterior cerebral vein (ACeV), prosencephalic artery (PrA), dorsal ciliary vein (DCV), middle cerebral vein (MCeV) (Figure 3.12. – D, E, F).

Once again, we accompanied *cup* control development at 96 hpf. At this stage, most changes observed in vasculature consisted of the increase of length and number of digestive system and intracranial vessels. While clear observation of head vessels was impaired for the same reason described at 72 hpf, we detected the basilar artery (BA), posterior communicating segment (PCS), primordial midbrain channel (PMBC), nasal vein (NV), anterior cerebral vein (ACeV), prosencephalic artery (PrA), dorsal ciliary vein (DCV) and middle cerebral vein (MCeV) (Figure 3.12. – G, H, I). Anew, at this stage we were able to observe the pectoral artery (PA), which originates from the dorsal aorta and takes blood into the forming fin ¹⁸⁷ (Figure 3.11. – F). Caudal vein plexus simplification and mouth protrusion were more advanced as well (Figure 3.11. – C, F, I).

Throughout this live temporal observation of *cup* controls some of the larvae died. We think that the main reason for this was the repeated mounting and dismounting in 2% methyl cellulose that was necessary to acquire images, which could inflict injury in embryos. Additionally, the use of MS-222 as anesthesia, also required for image acquisition, could ended up being toxic. Therefore, we observed less larvae at 72 (n=14) and 96 hpf (n=5) compared with that at 48 hpf (n=17).

It is also important to mention dsRed levels were more intense in *cup* control than non-injected, possibly due to the selection of embryos (offspring of *curly-up* (*cup*)^{tc321} crossing with *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2}) with higher expression of this signal that were grown for this line.



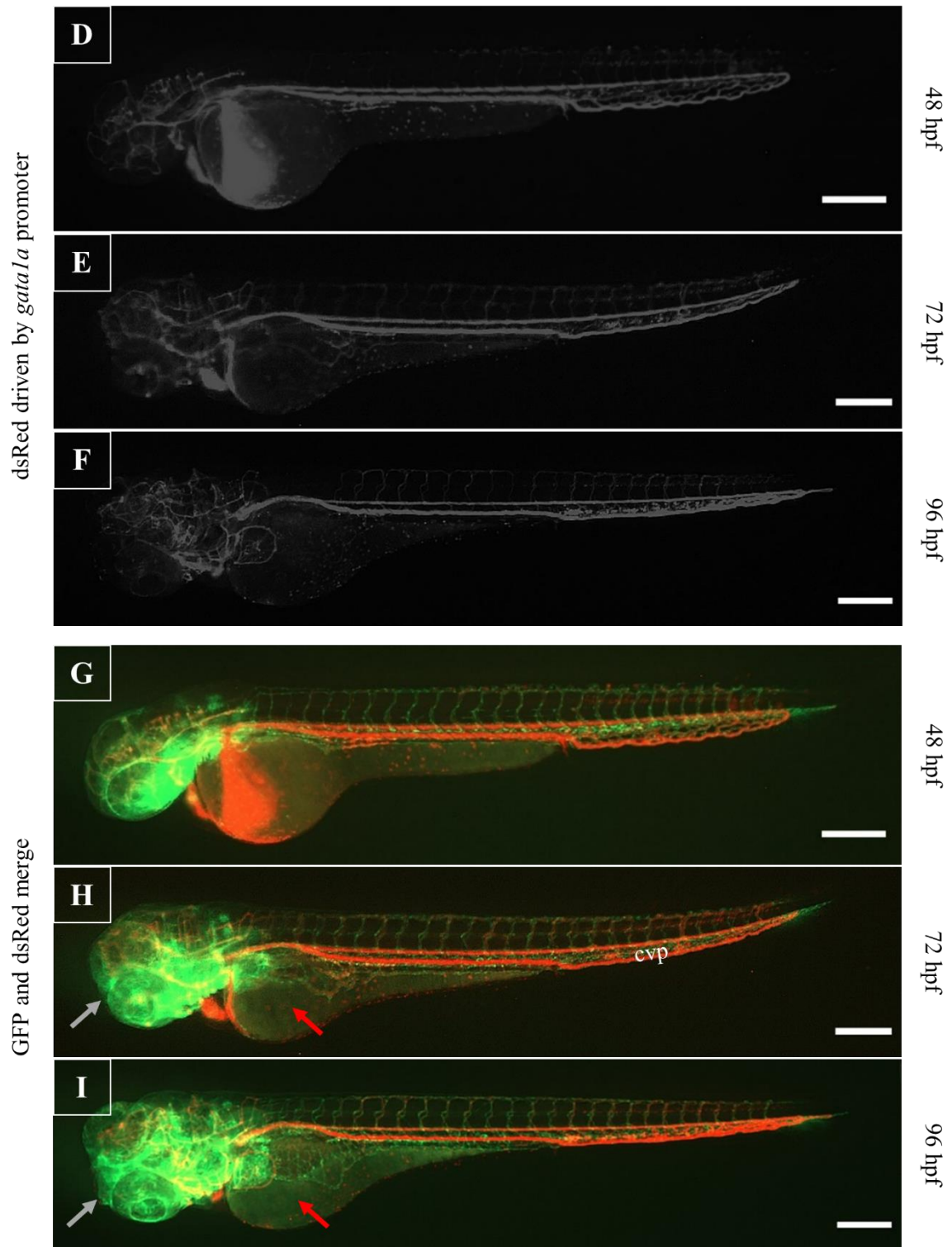


Figure 3.11. – Characterization of *curly-up* (*cup*)^{tc321} × *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2} mutant zebrafish line. Left lateral view of embryos at 48 hpf (A, D, G), 72 hpf (B, E, H) and 96 hpf (C, F, I) acquired by fluorescence stereomicroscopy (Zeiss Lumar V12). A), B) C) Expression of GFP under the control of the *fli1* promoter. D), E), F) Expression of dsRed under the control of *gata1a* promoter, tagging red blood cells. G), H), I) Merge of the expression of both GFP and dsRed. Scale bars: 250 μ m. Structures and vessels represented: caudal artery (CA), caudal vein (CV), caudal vein plexus (cvp), common cardinal vein (CCV), dorsal aorta (DA), dorsal longitudinal anastomotic vessel (DLAV), hepatic portal vein (HPV), intersegmental vessels (Se), pectoral artery (PA), suprainstestinal artery (SIA) and subintestinal vein (SIV). Grey arrows: mouth protrusion. Red arrows: smaller yolk than at 48 hpf.

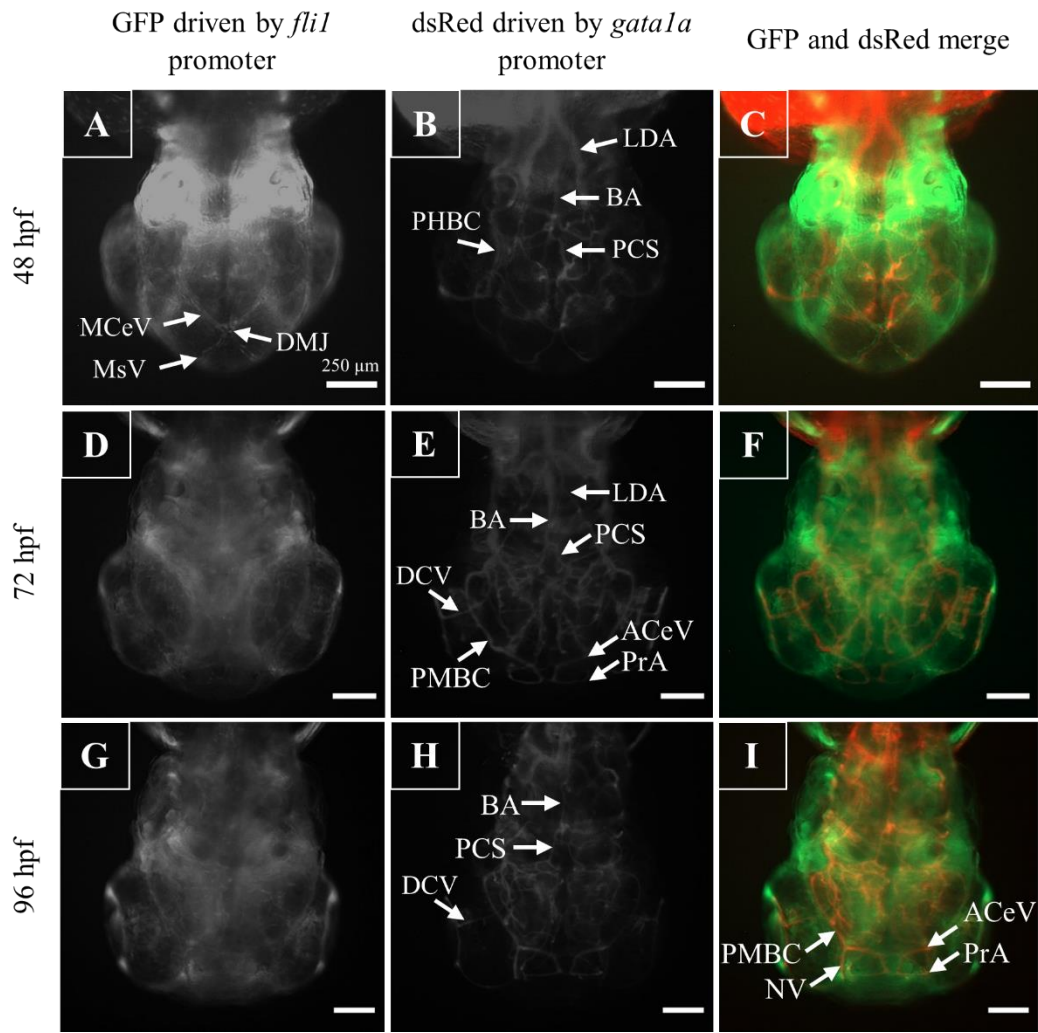


Figure 3.12. – Characterization of *curly-up* (*cup*)^{tc321} × *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2} mutant zebrafish line. Dorsal view of embryos at 48 hpf (A, B, C), 72 hpf (D, E, F) and 96 hpf (G, H, I) acquired by fluorescence stereomicroscopy (Zeiss Lumar V12). A), D), G) Expression of GFP under the control of the *fli1* promoter. B), E), H) Expression of dsRed under the control of *gata1a* promoter, tagging red blood cells. C), F), I) Merge of the expression of both GFP and dsRed. Scale bars: 100 μm. Structures and vessels represented: anterior cerebral vein (ACeV), basilar artery (BA), dorsal ciliary vein (DCV), dorsal middle junction (DMJ), lateral dorsal aorta (LDA), middle cerebral vein (MCEV), mesencephalic vein (MsV), metencephalic artery (MtA), nasal vein (NV), posterior communicating segment (PCS), primordial hindbrain channel (PHBC), prosencephalic artery (PrA).

3.4. Impact of *cup* mutation

The curly-up (*cup*)^{tc321} × *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2} mutant zebrafish line, allowed us to overcome morpholino limitations, such as, effectiveness estimation of knockdown, possible off-target effects and difficulty in precise, reproducible morpholino embryo injection²¹². However, this line in particular has two major drawbacks. First, as has been documented for the original *cup*^{tc321}, the curly-up (*cup*)^{tc321} × *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2} mutant zebrafish line is expected to show a maternal contribution of the *pkd2* mRNA^{172,213} and, thus, protein¹⁷⁴ for at least its first 36 hpf. Second, mutant lines have been described to may suffer genetic compensation¹⁸¹. With the aim of finding if *Pkd2* loss is associated with the vascular anomalies at later developmental stages, we analyzed by live stereomicroscopy (Zeiss Lumar V12) embryos and larvae *cup*^{-/-} and compared them with their *cup*^{+/+} and *cup*^{+/-} siblings.

3.4.1. Characterization of zebrafish defects

Even though we didn't analyze as many *cup*^{-/-} mutants (n=17) as we did *pkd2*-morphants (n=25), contrary to what was expected, *cup*^{-/-} mutant embryos did not show many defects. In fact, among those that were evident, some only appeared in later stages of development and were transient, not being posteriorly detectable. From our analysis, the defects that were persistent were the dorsal axis curvature and cardiac edema.

Starting once again with the same feature, homozygous embryos to the *cup*^{tc321} mutation were screened by the presence of the upwards dorsal axis curvature¹⁷², which, as mentioned above, is a phenotype associated with *Pkd2* loss in zebrafish (Figure 3.13.). However, when observing the expression of dsRed driven by *gata1a* promoter, and in contrast to *pkd2*-morphants, *cup*^{-/-} mutants blood flow was never compromised, and erythrocyte circulation was never absent from any analyzed vessel. Moreover, we did not observe malformation of the caudal vein plexus or incomplete intersegmental vessels, during the analyzed developmental stages of the *cup*^{-/-} mutants.

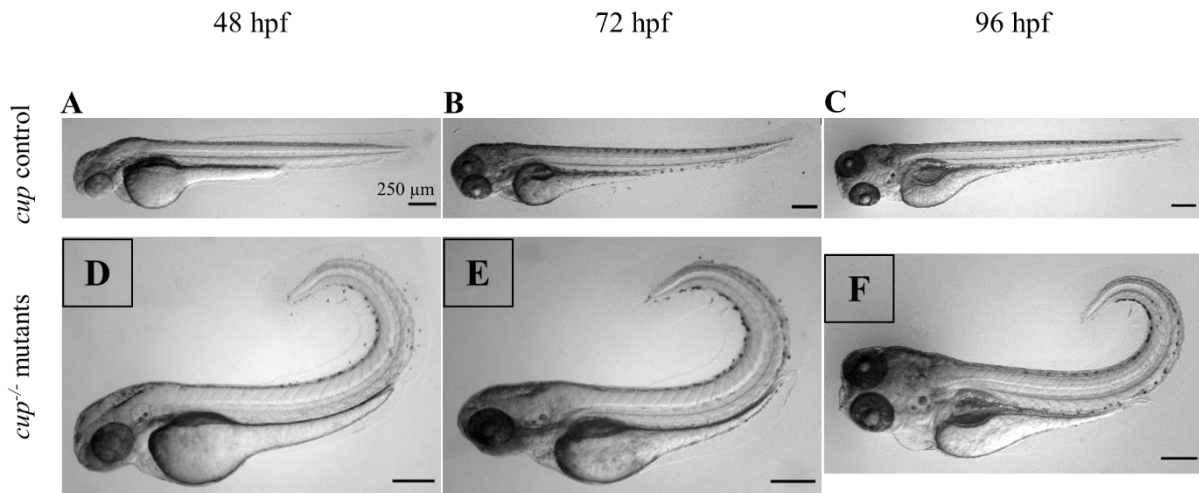


Figure 3.13. – Dorsal axis curvature (curly-up) in *cup*^{-/-} mutants. Left lateral view of embryos and larvae at 48 hpf (A, D), 72 hpf (B, E) and 96 hpf acquired by brightfield stereomicroscopy (Zeiss Lumar V12). A), B), C) *cup* control (*cup*^{+/+} or *cup*^{+/-}). D), E), F) *cup*^{-/-} mutant. Scale bars: 250 μ m.

Cardiac edema was increasingly observed throughout development. At 48 hpf, it affected only 6.7% of *cup*^{-/-} mutants, existing no statistically significant association between this defect and the loss of Pkd2 ($p=0.6130$). As the control group includes heterozygous individuals for the *cup*^{tc321} mutation, we may speculate that the lack of 50% of the functional protein could already be enough to cause cardiac edema in some individuals, which may contribute to part of the frequency of cardiac edema observed in *cup* controls at 48 hpf (11.8%). However, cardiac edema aggravated overtime, affecting 33.3% and 100% of *cup*^{-/-} larvae at 72 and 96 hpf, respectively (Figure 3.14.). Once again, applying Fisher's test, to compare *cup*^{-/-} mutants with *cup* control, we found a statistically significant association between the loss of Pkd2 and cardiac edema at both embryonic developmental stages ($p=0.0171$ and $p=0.0005$, respectively). This suggested that Pkd2 maternal contribution is the main reason for the initial attenuation of cardiac edema, and its progressive silencing results in a decrease of Pkd2 and in the aggravation of this phenotype throughout embryonic development of the heart. Heart laterality scoring was not performed for these embryos as we started our observations at 48 hpf, however it has been previously reported to occur in 33% of *cup*^{-/-} mutants¹⁷².

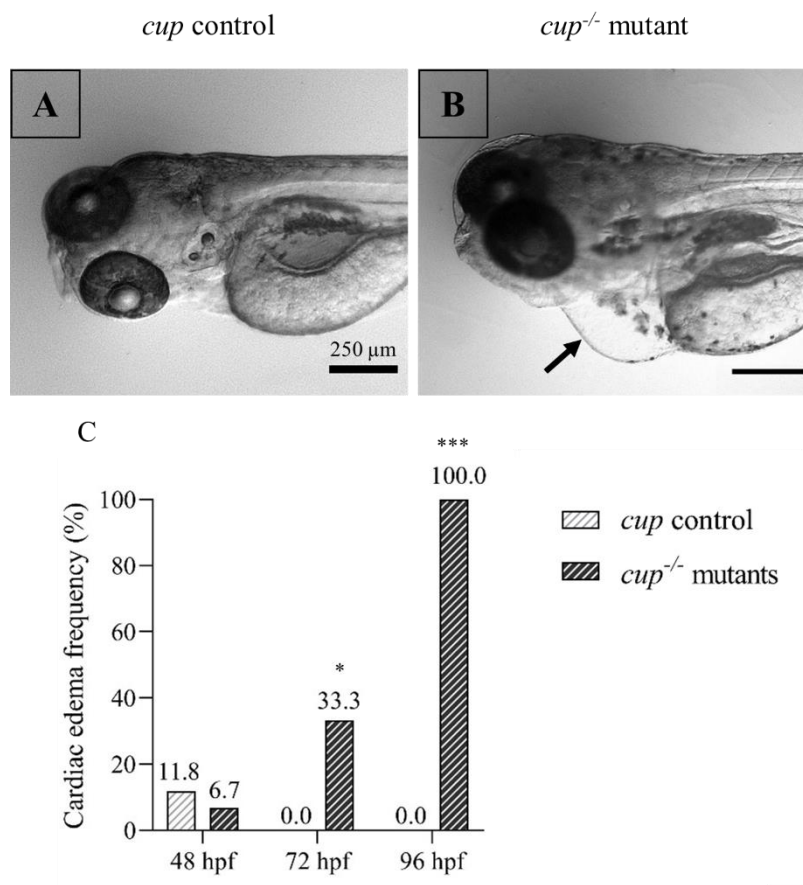


Figure 3.14. – Cardiac edema in *cup*^{-/-} mutants. Left lateral view of a *cup* control (A) and *cup*^{-/-} mutant at 96 hpf acquired by brightfield stereomicroscopy (Zeiss Lumar V12). Black arrow: cardiac edema. Scale bar: 250µm. D) Frequency of occurrence of cardiac edemas in *cup* control (n=17, n=14, n=5) and *cup*^{-/-} mutants (n=30, n=27, n=5) at 48, 72 and 96 hpf. * $p<0.05$, *** $p<0.001$, meaning a significant association between the loss of Pkd2 and cardiac edema, by Fisher's-test.

As mentioned before, the presence of hemorrhages is indicative of vascular problems. Although we did observe this anomaly at 48 hpf (in *cup* control) and 72 hpf (in *cup*^{-/-} mutants) (Figure 3.15.), these associations were not statistically significant ($p=0.3617$ and $p=0.5390$, respectively). Upon following these embryos and larvae at later stages, they did not present these abnormal accumulations of erythrocytes outside of the vessels. However, the lack of a statistical significant association of Pkd2 loss with this phenotype might be due to not its transitoriness but to the small number of observations at 96 hpf ($n=9$).

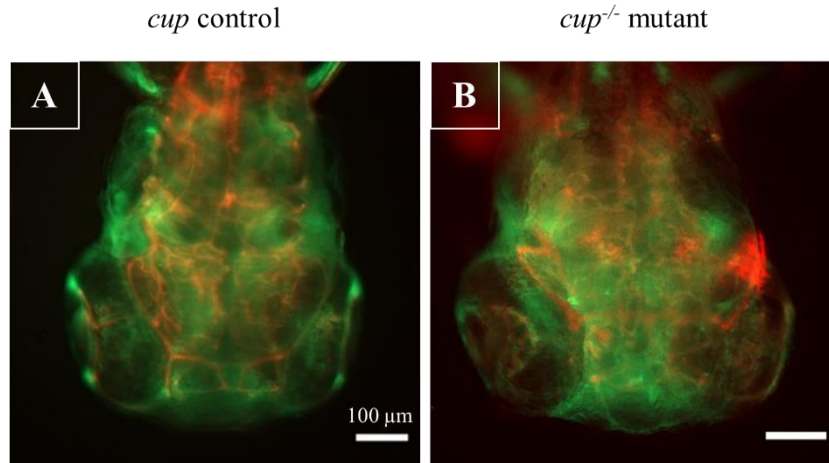


Figure 3.15. – Intracranial hemorrhage in *cup*^{-/-} mutants. Dorsal view of merge GFP driven by *fli1* promoter and dsRed by *gata1a* promoter expression of a *cup* control (A) and *cup*^{-/-} mutant's (B) head at 72 hpf acquired by fluorescence stereomicroscopy (Zeiss Lumar V12). White arrow: hemorrhage. Scale bars: 100 μ m.

We also encountered what appeared to be a “cloud” of erythrocytes outside of cerebral blood vessels, in 72 and 96 hpf *cup*^{-/-} mutants' larvae. However, such characteristic was also very present in *cup* control of the same age, which made us to question whether this was indeed a defect or if it was a normal accumulation due to large vessels, or several small vessels close together (Figure 3.16). It could as well be that this phenotype develops once Pkd2 decreases below its critical threshold, and that our observations in *cup* control were of heterozygous *cup*^{+/-} larvae. Or even that with growing complexity of the cerebral vasculature, the stereoscope's resolution is not enough for us to be able to clearly distinguish phenotypes, which might induce in error our observation. Therefore, going further, it is imperative to make the same experiment with equipment that allows higher amplification and resolution. Lastly, *cup*^{-/-} mutants never developed hydrocephalus.

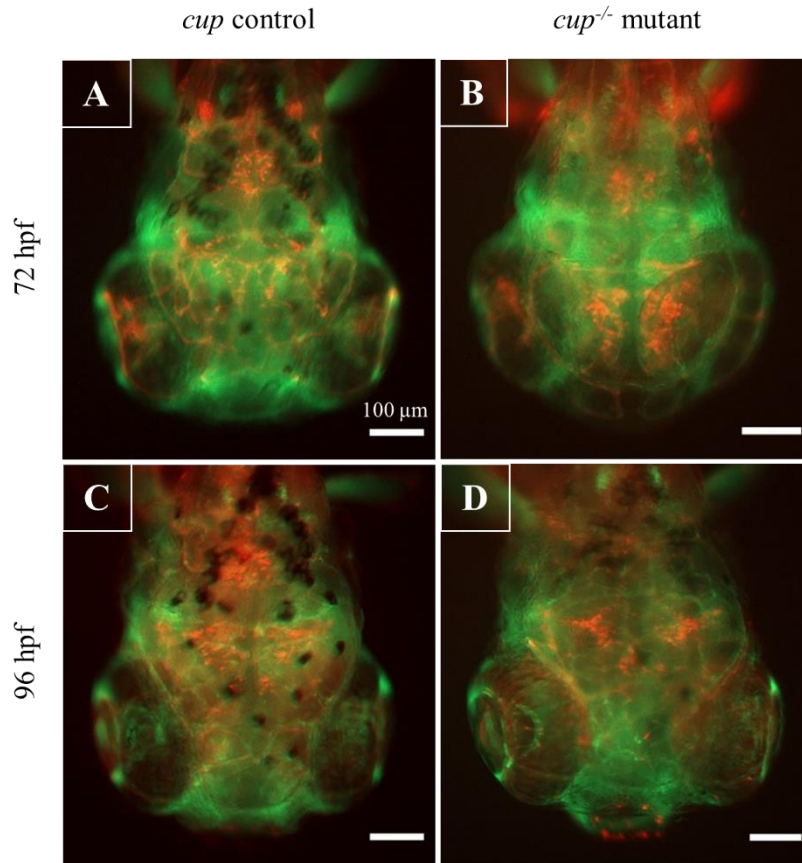


Figure 3.16. – “Cloud” of erythrocytes outside of blood vessels in *cup*^{-/-} mutants’ head. Dorsal view of merge GFP driven by *fli1* promoter and dsRed by *gata1a* promoter expression in *cup* control (A, C) and *cup*^{-/-} mutants’ larvae (B, D) at 72 hpf (A, B) and at 96 hpf (C, D) acquired by fluorescence stereomicroscopy (Zeiss Lumar V12). White arrows: “cloud” of erythrocytes. Scale bars: 100 μ m.

3.4.2. Dorsal aorta diameter

Like we did for *pkd2*-morphants, *cup* control and *cup*^{-/-} mutants’ dorsal aorta was measured at the same 5 locations: 5th, 10th, 15th, 20th and 25th myotome, using the ImageJ software. This time however, we were able to accompany the progression of this vessel diameter post 48 hpf, after which it starts decreasing²⁰⁹. This reduction may be driven by changes in endothelial cells, in response to shear stress, which elongate in the flow direction²¹⁴. Indeed, we were able to observe this throughout embryogenesis (Table 3.2.).

Table 3.2. – Mean and standard deviation (SD) of dorsal aorta diameter measurements at the 5th, 10th, 15th, 20th and 25th myotome and number (n) of analyzed *cup* control and *cup*^{-/-} mutants at 48, 72 and 96 hpf.

			5 th	10 th	15 th	20 th	25 th
48 hpf	<i>cup</i> control	Mean (μ m)	26.294	25.887	25.376	22.567	19.233
		SD (μ m)	2.126	2.022	2.257	2.588	2.004
		n	17	17	17	17	17
	<i>cup</i> ^{-/-} mutants	Mean (μ m)	20.808	22.011	20.173	18.258	15.605
		SD (μ m)	3.290	2.887	2.731	2.518	2.663
		n	30	30	29	29	29

72 hpf	<i>cup</i> control	Mean (μm)	21.365	21.133	20.486	18.386	14.816
		SD (μm)	2.799	2.085	2.513	2.862	1.569
		n	14	14	14	14	14
	<i>cup</i> ^{-/-} mutants	Mean (μm)	17.431	19.197	17.668	16.023	13.214
96 hpf	<i>cup</i> control	SD (μm)	2.182	3.256	2.711	2.400	2.056
		n	28	28	28	28	28
		Mean (μm)	17.794	19.034	17.581	16.392	14.825
		SD (μm)	1.937	1.206	1.900	1.496	1.329
		n	5	5	5	5	5
	<i>cup</i> ^{-/-} mutants	Mean (μm)	15.449	18.706	16.563	14.367	10.946
	<i>cup</i> ^{-/-} mutants	SD (μm)	1.761	2.037	3.160	1.704	0.865
		n	9	9	9	9	9

Our analysis of *cup*^{-/-} mutants at 48 hpf, showed that, diameter was always significantly narrower than *cup* control embryos' ($p < 0.0001$) (Table 3.2. and Figure 3.17.), similarly to what we observed in *pkd2*-morphants. However, these differences were unexpectedly lost throughout development. Indeed, at 72 hpf, significant differences were only found at the 5th, 15th and 20th and 25th segment ($p < 0.0001$, $p = 0.0023$, $p = 0.0074$ and $p = 0.0143$, respectively). And, at 96 hpf the only observed significant differences at the 5th, 20th and 25th ($p = 0.0396$, $p = 0.0467$ and $p < 0.0001$) (Figure 3.17.).

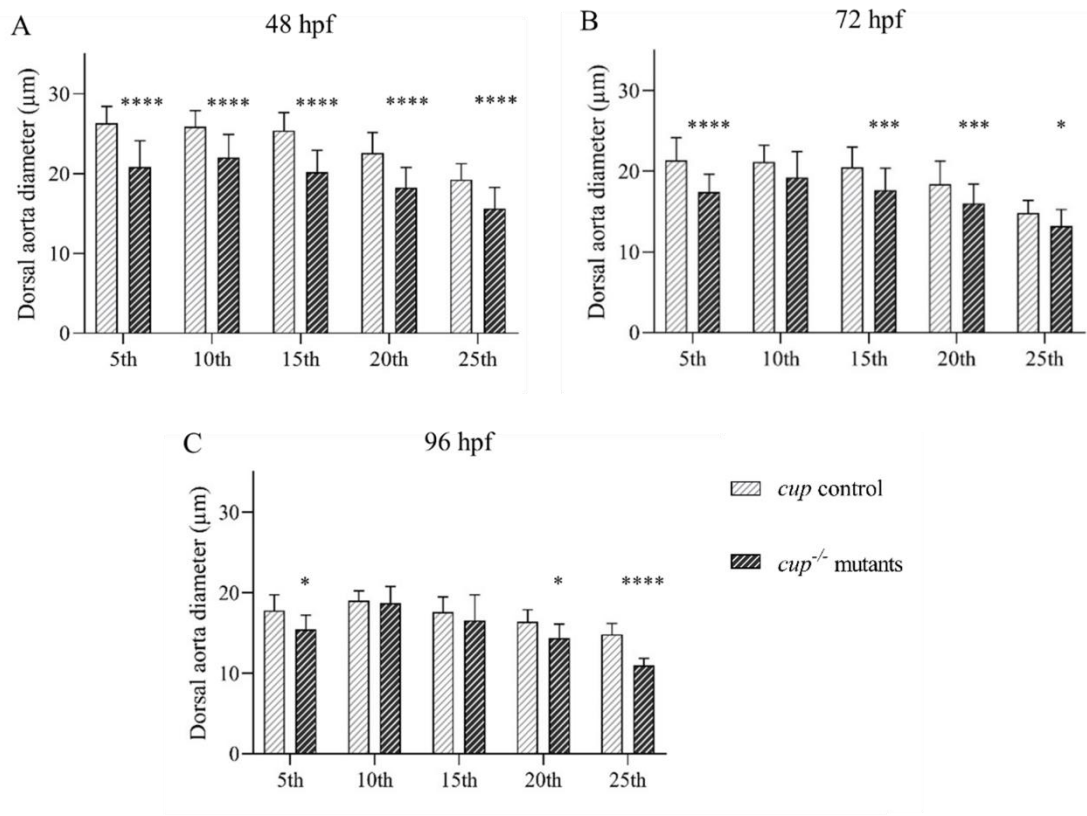


Figure 3.17. – Dorsal aorta diameter (μm) of 48 (A), 72 (B) and 96 hpf (C) *cup*^{-/-} mutants at the 5th, 10th, 15th, 20th and 25th myotome. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ significantly different from *cup* control by Mann-Whitney *U* test and Student's *t*-test. *cup*^{-/-} mutants' 5th measurements at 48 and 72 hpf do not follow a normal distribution; remaining measurements at all developmental stages follow a normal distribution with equal variances. As such, Mann-Whitney *U* test was used to compare 5th measurements at 48 and 72 hpf between *cup*^{-/-} mutants and *cup* control; Student's *t*-test was used to compare all remaining measurements between *cup*^{-/-} mutants and *cup* control at all developmental stages.

Based on our results, we can postulate that possibly Pkd2 holds a more determinant role in the vasculature at earlier stages of embryogenesis. We mentioned that, in *cup*^{-/-} mutants, blood flow never seemed to be slower or otherwise impaired, so we can imagine that mechanical cues might be sufficient to vascular remodeling after 48 hpf. However, we can't discard the possibility of Pkd2 importance at 72 and 96 hpf, especially given the low number of larvae analyzed at the latter (n=9). Therefore, more experiments should be done in order to better understand this subject.

3.5.A peek with higher resolution

From the beginning, our intention was to fully characterize and observe possible vascular alterations associated with the lack of Pkd2 in higher resolution by confocal microscopy (Zeiss LSM 710), particularly intracranial vasculature as it was less clear in Zeiss Lumar V12. It was not possible due to time limitations, however we were able to satisfy our curiosity by analyzing a few embryos at 48 hpf, using a fluorescence microscope (Zeiss Observer) that would allow us higher resolution for each z position.

Taking the opportunity to see these vessels in a three-dimensional manner, we analyzed 2 embryos at 48 hpf of *pkd2*-morphants, mismatch-control, non-injected, *cup* control and *cup*^{-/-} mutants. Although it would be preferable to obtain *in vivo* images, it proved to be challenging due to dorsal axis curvature. As such, embryos had to be fixed in 4% PFA during 24 hpf, after which they were severed immediately after the yolk, in order to facilitate mounting of the head in 2% (w/v) low-melting agarose.

Our observations didn't diverge from what we already described for each experiment at 48 hpf. Indeed, the presence of hemorrhages in *pkd2*-morphants was similar to what we observed before. We noticed that we were not able to detect dsRed in the same way as *in vivo*, many times not even being detected. This is something that could be related to photobleaching, but also it is possible that fixation did not occur quickly enough to fixate erythrocytes exactly as they circulate *in vivo*. Therefore, it is important to develop an embryo mounting method of zebrafish with curly-up, that will allow *in vivo* observation.

Figure 3.18. shows our preliminary confocal microscopy analysis of a single *pkd2*-morphant (previously observed in Zeiss Observer) that presented a hemorrhage as well as an accumulation of erythrocytes in the middle cerebral vein (MCeV). Focusing on the latter we were able to see a small bulb dilation of this vessel, which could explain this happening. Most importantly, this very brief sneak peek showed what a great tool it could be to characterize neurovascular defects associated with the lack of Pkd2 and to clarify all doubts that emerged during our analysis.

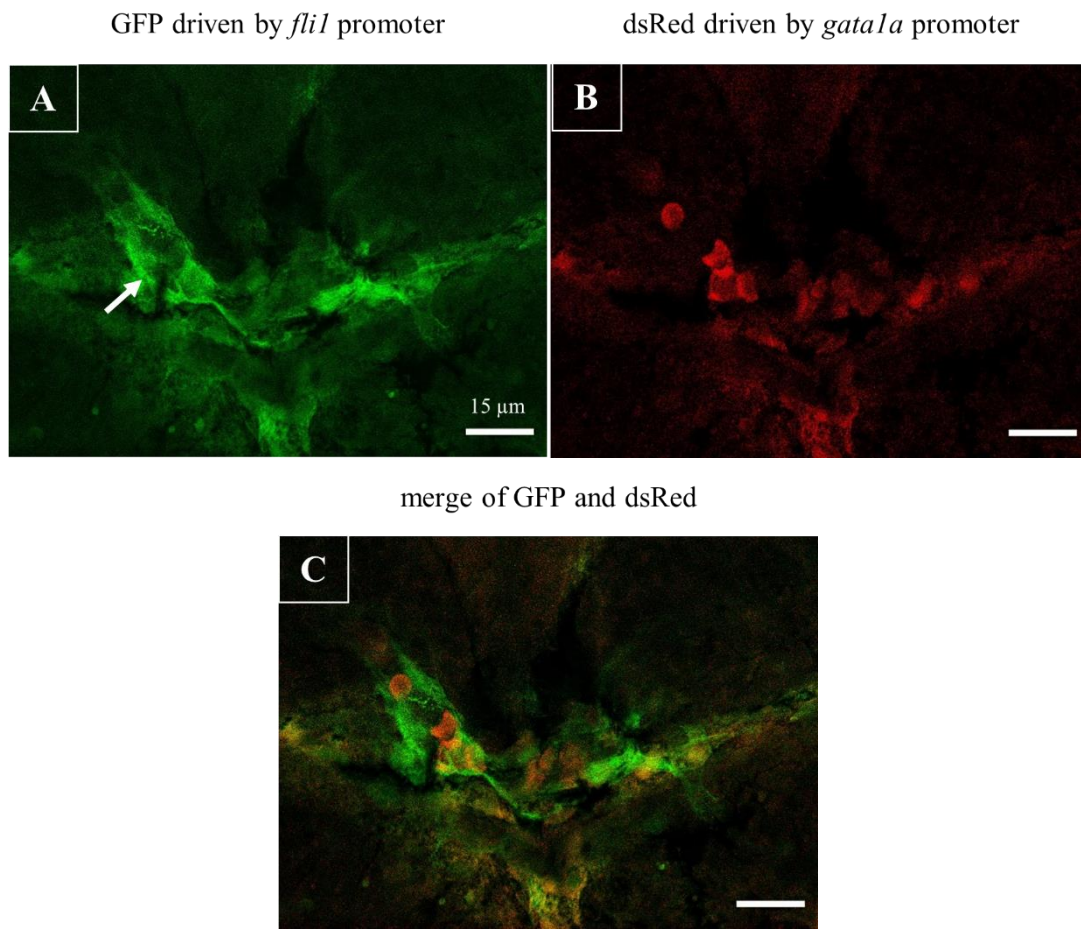


Figure 3.18. – Possible bulb dilation of the middle cerebral vein (MCeV) of *pkd2*-morphant. Head dorsal view of a *pkd2*-morphant at 48 hpf acquired by fluorescence confocal microscopy (Zeiss LSM 710). A) Expression of GFP driven by *fli1* promoter. B) Expression of dsRed driven by *gata1a* promoter. C) Merge of the expression of both GFP and dsRed. White arrows: vessel dilation. Scale bars: 15 μ m.

4. DISCUSSION

Although ADPKD is clinically characterized by the development of several fluid-filled cysts, which gradually impair kidney parenchyma and function, this disease is not limited to this organ. Indeed, it manifests in several organs and systems, leading to many more complications. This project was focused on vascular abnormalities associated to ADPKD, which can lead to intracranial aneurysms, aorta dissection and hypertension ³². Vascular conditions that majorly contribute to disease progression and cardiovascular complications, which are the leading cause of morbidity and mortality in these patients ¹²⁸.

Evidence from literature has shown that PC2 is widely expressed in the endothelial cells of mesenteric arteries ²¹⁵ and in the smooth muscle cells of cerebral arteries ⁵⁹, aorta and mesenteric arteries ^{53,216}, suggesting an important function of this protein in the cardiovascular system. The contribution of the PC2 channel to vascular changes observed in ADPKD remain unclear. Indeed, it appears that PC2 regulation and function varies in different vascular beds ¹³⁸.

Knowing that this polycystin sequence and function in zebrafish is conserved with human ortholog ¹⁷⁶, we thought that this animal would be a good model to study the relevance of Pkd2 in the vasculature. Using embryos from zebrafish lines *Tg(fli1:eGFP; gata1a:dsRed)^{y1;sd2}*, for *pkd2* knockdown, and *curly-up (cup)^{tc321} × Tg(fli1:eGFP; gata1a:dsRed)^{y1;sd2}*, a *pkd2* mutant, we postulated that the loss of Pkd2 would lead to vascular anomalies. In particular, we expected to observe alterations similar to those observed before by our group and others in *pkd2*-morphant zebrafish such as body axis curvature, organ laterality defects, cardiac edema ¹⁷⁸ and hydrocephalus ¹⁷³. As well as possible aneurysm-like dilations in intracranial vasculature and hemorrhages as seen in *Pkd2^{+/-}* mice ¹³⁶.

There are many studies of vascular embryonic development in zebrafish, that properly identify vessels. We based our vasculature descriptions of both zebrafish lines on “The Interactive Atlas of Zebrafish Vascular Anatomy” from Weinstein lab ²⁰¹, that have extensively characterized zebrafish vasculature throughout embryonic development. This was an extremely helpful source to properly characterize vasculature in this line. It is important to caveat that, in our experiments, the identification of embryo vessels was only highlighted if their observation was very clear, given the resolution limitations.

To complete this analysis, we intended to further characterize intracranial vasculature and possible alterations through confocal microscopy (Zeiss LSM 710). However, it was not possible to produce very preliminary data due to time limitations.

4.1. Cardiovascular impact of *pkd2* knockdown

Firstly, we thought of using *Tg(fli1:eGFP)^{y1}* zebrafish line to observe embryo's vasculature. However, it lacked for proper observation of intracranial vessels and of defects such as hemorrhages or blood circulation alterations. Nonetheless, it was important because it allowed us to identify and troubleshoot several drawbacks of optimal image quality, such as the development of pigmentation and the movement of the embryos during observation. To overcome the first problem, we decided to incubate embryos with 200 µM PTU. Although PTU is toxic, the concentration used appeared to have no effect in all embryos observed. And, the second drawback was overcome by larvae incubation with MS-222 (0.16 mg/mL) solution in E3 medium.

On the other hand, *Tg(fli1:eGFP, gata1:dsRed)^{y1:sd2}* zebrafish line was extremely useful to characterize zebrafish vasculature. Besides all the advantages associated with the use of zebrafish as a model, the observation of GFP and dsRed expression, which tagged zebrafish endothelium and erythrocytes, respectively, in Zeiss Lumar V12 was a great way to study vascular anomalies in a high throughput manner. We noticed that at 24 hpf, there was already a strong GFP expression under the control of *fli1* promoter. However, it is important to mention that dsRed signal was not that strong and was difficult to detect. Since dsRed is expressed under *gata1a* promoter thus tagging erythrocytes, this could be because between 12 and 24 hpf zebrafish undergoes the first wave of primitive hematopoiesis. Therefore, at 24 hpf primitive erythrocytes are only beginning to enter circulation and mature ²¹⁷, which could have diffculted signal detection. However, this drawback was overcome at 48 hpf as we detected a clear dsRed signal. It is, also, important to mention that the intensity of the *fli1* promoter-driven GFP and *gata1a* promoter-driven dsRed signals were variable among the embryos and that the yolk of zebrafish embryos and larvae is naturally auto-fluorescent, which diffculted our analyses.

Comparing our results of 48 hpf non-injected with *pkd2*-morphants, our analysis was coincident with what was expected. Indeed, major statistically significant defects involving cardiovascular alterations were detected: heart situs defects, cardiac edema, hemorrhages and hydrocephalus, as well as occasional alterations, such as the malformation of the caudal vein plexus, incomplete formation of intersegmental vessels.

Both caudal vein plexus and intersegmental vessels defects observed were not significantly associated with the lack of Pkd2, suggesting a morpholino off-target effect or possibly a slight delay in development associated with morpholino use. However, it is noteworthy to mention that both vessels are formed through angiogenesis in a process that seems to depend on ERK5-Klf2a-Nos2b ²¹⁸ and both VEGF and Notch ¹⁶⁵ signaling, respectively. It is known that Notch signaling is involved in left-right patterning in zebrafish ²¹⁹ and that *pkd2*-knockdown causes laterality defects, which means that some of these signaling pathways might be affected by Pkd2 loss. Something that could potentially be further looked upon by quantifying mRNA transcripts of both VEGF and Notch, through RT-PCR. Caudal vein plexus malformation could also be attributed to impaired blood flow, which regulates angiogenesis of this vessel also through ERK5-klf2a-nos2b signaling ²¹⁸.

As mentioned before, zebrafish *pkd2*-morphants and *cup*-mutants present heart laterality defects, which is caused by impairment of Pkd2 and fluid flow in the KV, that similarly affect *dand5* expression pattern and organ situs ²⁰⁵. This feature has been extensively quantified by our group ²⁰⁵. From our results we could see that there was a significant association between heart situs defect with loss of Pkd2, however the percentage of occurrence was lower (16.7%) than expected (32%) which may be explained by the low number of observations in the present work. Also, it is related to Pkd2 function in the KV and not on the vasculature, therefore, for cardiac anomalies we focused on observed edema.

Our results showed that cardiac edema was highly penetrant in *pkd2*-morphants (56%) and that, indeed, the lack of Pkd2 is associated with this phenotype. Severe cardiac edema can be provoked by environmental stress as well as genetic changes, particularly during a critical period in heart development, which can culminate in heart failure and circulatory collapse ²⁰⁶. Paavola and his team have shown that zebrafish *pkd2* mutation led to slowed Ca²⁺ cycling in the heart and predispose to dilated cardiomyopathy. Furthermore, these zebrafish showed low cardiac output and atrioventricular block ¹⁷⁸. Besides impaired Ca²⁺ cycling, cardiac edema can result from atrioventricular problems ²²⁰. Consistent with this study, in our observations of *pkd2*-morphants, blood seemed to concentrate around the heart and blood circulation seemed impaired all throughout the body, as suggested by an accumulation of dsRed tagged erythrocytes, being much slower than normal and some of the times not even detected in the head of *pkd2*-morphants.

Besides this, major characteristics that evidence intracranial vascular problems are hemorrhages in the head and hydrocephalus. When testing if hemorrhages in *pkd2*-morphants were associated with loss of Pkd2 it was evident that they were significantly related. However, the percentage of hemorrhages' occurrence in mismatch-control raised suspicion that this defect was observed due to toxicity of the MO. To discard possible morpholino toxic effects, we also tested whether there was a significant association between the hemorrhages and the use of the *pkd2*-mismatch-MO when compared to the non-injected embryos. We found that there was not a significant association between them, which supports that this defect was indeed related to Pkd2 loss. We may speculate that the hemorrhages we observed originated from a ruptured aneurysm, caused by an increase of production and accumulation of extracellular matrix proteins, such as collagen III and fibrillin, increased proliferation of vascular smooth muscle cells and elastin fragmentation ¹⁴⁵. Similar to what has been done in *Pkd1*^{nl/nl} mice to understand PC1 implication in the maintenance of the vessel wall structural integrity ²²¹, we could use antibodies for fibrillin 2b, fibronectin, α -smooth muscle actin (*acta2/asma*), collagen II and XI to locate these proteins, through immunohistochemistry, in the vasculature of *pkd2*-morphants, as they have been implied in aneurysm formation and extracellular matrix response. This would give us an insight over disrupted protein expression that could be the source for this phenotype and other vascular manifestations of ADPKD.

On its turn, hydrocephalus was the most frequent vascular anomaly detected in *pkd2*-morphants and has also been shown to be significantly associated with Pkd2 loss in zebrafish ¹⁷³.

To complete the study of the *pkd2*-morphants, in a near future it will be necessary to further verify *pkd2*-augMO specificity by mRNA phenotype rescue experiments, of the caudal vein plexus malformation, incomplete intersegmental vessel formation, heart *situs* defects, cardiac edema, intracranial hemorrhages and hydrocephalus. This would help determine whether these phenotypes are more severe because *pkd2* knockdown inhibits both maternal and zygotic transcripts.

4.2. Cardiovascular impact of *cup* mutation

The generated *curly-up* (*cup*)^{tc321} $\times$ *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2} zebrafish line proved to be a great model to study zebrafish vasculature with Pkd2 loss. Indeed, it allowed us to overcome MO associated limitations such as the possible off-target effects, the difficulty in precise, reproducible morpholino embryo injection and the window of knockdown effectiveness. The latter reduces with time, not being effective from 72 hpf onwards, because MOs are asymmetrically distributed (causing dilution), due to distinct mitotic activity in different cellular lineages, and because they are eventually degraded overtime ²¹². Therefore, our time window of observation was extended. Moreover, dsRed levels were more intense in *curly-up* (*cup*)^{tc321} $\times$ *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2}, possibly because, when embryos from *curly-up* (*cup*)^{tc321} crossing with *Tg(fli1:eGFP; gata1a:dsRed)*^{y1;sd2} were grown for this line, we selected those who presented higher expression levels of both GFP and dsRed.

While interpreting these results, it is important to have in mind that our *cup* control group included both *cup*^{+/+} and *cup*^{+/-} embryos/larvae. Pkd2 haploinsufficiency can be enough to cause vascular alterations, therefore our control group does not translate completely into a typical embryonic development. This could have been tackled by genotyping observed embryos for the *cup*^{tc321} mutation through PCR, after image acquisition.

Contrary to what we expected, during the analyzed developmental stages, many *pkd2*-morphant phenotypes were not observed in *cup*^{-/-} mutants or were not significantly associated with lack of Pkd2.

We didn't detect malformation of the caudal vein plexus, or the incomplete formation of intersegmental vessels, and erythrocyte circulation suggested that blood flow was never compromised or absent from any vessel. The same was verified for intracranial hemorrhages and hydrocephalus.

It seems that, from our analysis, cardiovascular defects were mainly cardiac edema, a condition that aggravated, increasing in frequency and severity, throughout embryonic development. We know from Paavola work that, indeed, these *cup*^{-/-} mutants have low cardiac output and atrioventricular block¹⁷⁸, which could be attributed to defects in the mitral valve and reduced aorta compliance^{222,223}. Since, in humans, affected valvular tissue revealed defective collagen synthesis and myxomatous degeneration¹⁴⁷, and vascular smooth muscle cells, where *PKD2* is expressed, are responsible for providing collagen synthesis¹³⁹, we can postulate that *Pkd2* disruption may have an important role in the altered collagen synthesis mechanisms that lead to cardiac edema in zebrafish. We could assay whether defective collagen synthesis occurs in the heart of *cup*^{-/-} mutants by performing *in situ* hybridization using probes for different collagen genes present in zebrafish such as *col11a1a*, ortholog to human *COL11A1*²²⁴ which is expressed in heart valves²²⁵. In fact, this could be done for both *pkd2*-morphants and *cup*^{-/-} mutants and respective controls. Nonetheless, this mechanism might not underly all vascular phenotypes observed and *pkd2* might have unique influence on collagen gene regulation in different tissues. Nonetheless, Fisher's test analysis, at all tested stages of development, revealed that *cup*^{-/-} mutants' cardiac edema is associated with *Pkd2* loss.

The reasons behind the absence of some vascular defects in *cup*^{-/-} mutants, that were observed in *pkd2*-morphants, can be attributed by either off-target effects of *pkd2*-augMO in *pkd2*-morphants or maternal contribution of *pkd2* mRNA present in *cup*^{-/-} mutants¹⁷², which overcomes defective phenotypes. We believe that this may be the main contributor to our observations, given that as the *Pkd2* maternal contribution was progressively silenced, cardiac edema was aggravating throughout embryonic development of the heart. Indeed, our group has shown that *Pkd2* protein was present in pronephros cilia at least until 36 hpf¹⁷⁴. Our doubts could be tackled by immunolocalization assays of *Pkd2* in *pkd2*-morphants and *cup*^{-/-} mutants at different stages of development since fertilization. A better approach would be to perform Western-blot analysis, however, all the commercially available antibodies against mammalian *Pkd2* that our group have tested failed in detecting the protein by this technique¹⁷⁴.

It is important to mention that although this mutant can develop pronephric dilations, they never bud off from the tubules and never become true cysts¹⁷², contrary to *pkd2*-morphants that do¹⁷³. Zebrafish pronephros is functional from 76 hpf²²⁶, and studies have suggested that renin²²⁷ and the renin-angiotensin system (RAS)²²⁸ may be involved in ion homeostasis. We may speculate that perhaps RAS could also be involved in progression of other vascular anomalies. The development of dilations in *cup*^{-/-} mutants' pronephros could be enough to activate RAS and, as such, cause hypertension, which potentiates many of the vascular problems in ADPKD patients. Could be interesting to quantify renin in both *pkd2*-morphants and *cup*^{-/-} mutants.

In fact, it would be most advantageous to continue to observe *cup*^{-/-} mutants until 6 dpf, time at which *cup*^{-/-} die due to malformation complications, as it would allow us to observe a more prolonged effect of *Pkd2* loss. Even so, our results suggest that *Pkd2* may play a more important role in vessel development in the first 48 hpf, as we were able to see more defects induced by *Pkd2*-knockdown than in *cup*^{-/-} mutants.

4.3. Dorsal aorta diameter in *Pkd2* loss

Our results for dorsal aorta diameter measurements at 48 hpf were consistent in both *pkd2*-morphants and *cup*-mutants and were to be expected. Indeed, we can affirm that *pkd2* loss led to the exacerbated reduced caliber of this vessel throughout zebrafish embryo's body, possibly due to vasoconstriction. Our findings can be supported by Qian *et al.*, 2007. They made observations in *Pkd2*^{+/-} mice, that determined that Pkd2 disruption in vascular smooth muscle leads to an exaggerated vasoconstriction in response to phenylephrine stimulation in a Ca²⁺-independent manner¹⁸⁴. It would be interesting to understand if the molecular mechanisms that underly this vasoconstriction are similar to that verified by Qian *et al.*, 2007.

We were able to see that this vasoconstriction was more accentuated in *pd2*-morphants. When considering other vascular differences and defects associated with *pkd2* knockdown we highlight the impaired dsRed tagged erythrocytes circulation, possibly due to cardiac edema. Blood flow has an important role in vascular remodeling, which suggests that the blood flow alterations we observed might alter shear stress levels and lead to the decrease in blood vessel size²¹⁴. Also, as we have mentioned for previous cardiovascular manifestations, *cup*^{-/-} mutants might have an attenuated phenotype due to maternal mRNA contribution. Therefore, we could once again perform immunolocalization assays of Pkd2 in this vessel for *pkd2*-morphants and *cup*^{-/-} mutants at different stages of development since fertilization, since the dorsal aorta is the main embryonic artery along the trunk and is the first blood vessel to be perfused²²⁹.

The follow up of dorsal aorta diameter in *cup*^{-/-} mutants at 72 and 96 hpf, showed that the differences between mutants and control were decreasing and sometimes were not significant. It is known that post 48 hpf the diameter of dorsal aorta starts to decrease, driven by changes in endothelial cells, in response to shear stress, which elongate in the flow direction^{209 214}. Since we can not compare if the same occurs for *pkd2*-morphants because the effectiveness of the *pkd2*-morpholino is progressively lost over time, we can postulate that this could happen because Pkd2 holds a more determinant role in the vasculature at earlier stages of embryogenesis. We can imagine that since *cup*^{-/-} mutants' blood flow never seemed to be slower or otherwise impaired, mechanical cues independent of Pkd2 pathway might be sufficient for normal vascular remodeling after 48 hpf.

These results were very important because they proved that, if in *cup*^{-/-}, which maintain the curly-up tail, the diameter of the aorta normalizes throughout development, then it is because the decrease in the diameter of the aorta in the early stages (of both *pkd2*-morphants and *cup*^{-/-} mutants) is not due to the curvature itself.

However, the number of analyzed larvae at 72 and 96 hpf was low compared with others, which affects difference in significance and so we can't discard the possibility of Pkd2 importance at these developmental stages. More experiments should be done in order to increase this number of observations and to better understand this subject.

Overall, the observation of zebrafish through live-fluorescence stereomicroscopy (Zeiss Lumar V12) presented several advantages over confocal microscopy. The time necessary to prepare the embryos and larvae and acquire images was much lower, allowing us to not only observe more zebrafish but also to avoid photobleaching due to UV light exposure, which enabled a very thorough visualization of the entire vasculature development and anomalies. Nonetheless, in the future these alterations should be further characterized with higher resolution.

5. CONCLUSION

The main objective of this work was to evaluate if the lack of *pkd2* would lead to vascular alterations associated with ADPKD. Literature had shown that both PC1 and PC2 are expressed in the vasculature and the disruption of *pkd2* led to enhanced smooth muscle contractility and impaired endothelial-dependent vasorelaxation¹⁸⁴. However, the contribution of the PC2 channel to vascular changes observed in ADPKD remain unclear.

We then chose to use both *Tg(fli1:eGFP; gata1a:dsRed)^{y1;sd2}* and *curly up (cup)^{tc321}* x *Tg(fli1:eGFP; gata1a:dsRed)^{y1;sd2}* zebrafish lines to make our *in vivo* observations. With the experiments performed, we observed that *pkd2*-morphants presented several cardiovascular anomalies which can be linked to *pkd2* disrupted pathways, such as heart situs defects, cardiac edema, hemorrhages and hydrocephalus. However, not all phenotypes were found in *cup^{-/-}* mutants, even when observing further developmental stages. Cardiac edema was the main cardiovascular defect detected. This suggested that these embryos could have a significant maternal contribution, which can attenuate defective phenotypes. We believe that this may be the main contributor to our observations in *cup^{-/-}* mutants, given that as the Pkd2 maternal contribution was progressively silenced, cardiac edema was aggravating throughout embryonic development of the heart. This shows the need for further investigation of molecular mechanisms by which *pkd2* could influence vasculature. Indeed, we intended to perform immunolocalizations in zebrafish blood vessels of both *pkd2*-morphants and *cup^{-/-}* mutants, and respective controls, in order to better understand our findings, but it was not possible due to time limitations. The same was the case for further characterization of intracranial vasculature by confocal microscopy (Zeiss LSM 720).

Additionally, at 48 hpf dorsal aorta diameter measurements in both *pkd2*-morphants and *cup^{-/-}* mutants showed that this vessel had a significant reduced caliber, which seems to be caused by vasoconstriction. Such difference was not as significant or verified at later developmental stages in *cup^{-/-}* mutants which suggested either that Pkd2 had a more determinant role in the vasculature at earlier stages of embryogenesis or that the mechanical cues that regulate vascular remodeling are sufficient for normal vascular remodeling and may be independent of Pkd2 function. These results also showed that if in *cup^{-/-}*, which maintain the curly-up tail, the diameter of the aorta normalizes throughout development, then it is because the decrease in the diameter of the aorta in the early stages (of both *pkd2*-morphants and *cup^{-/-}* mutants) is not due to the curvature itself.

Most importantly, our work serves as a base for further research in the crosstalk between Pkd2 and several pathways, whose disruption cause vascular problems using the zebrafish line we generated: *curly up (cup)^{tc321}* x *Tg(fli1:eGFP; gata1a:dsRed)^{y1;sd2}* and possibly complementing this study with *pkd2* knockdown.

6. REFERENCES

1. World Health Organization. *The World Health Report 2002 - Reducing Risks, Promoting Healthy Life.*; 2002. doi:10.1080/1357628031000116808
2. Chapman AB, Devuyst O, Eckardt KU, et al. Autosomal-dominant polycystic kidney disease (ADPKD): Executive summary from a Kidney Disease: Improving Global Outcomes (KDIGO) Controversies Conference. *Kidney Int.* 2015;88(1). doi:10.1038/ki.2015.59
3. Abbafati C, Machado DB, Cislighi B, et al. Five insights from the Global Burden of Disease Study 2019. *Lancet.* 2020;396(10258). doi:10.1016/S0140-6736(20)31404-5
4. Dalgaard OZ. Bilateral polycystic disease of the kidneys; a follow-up of two hundred and eighty-four patients and their families. *Acta Med Scand Suppl* . 1957;328:1-255.
5. Lanktree MB, Haghighi A, Guiard E, et al. Prevalence estimates of polycystic kidney and liver disease by population sequencing. *J Am Soc Nephrol.* 2018;29(10). doi:10.1681/ASN.2018050493
6. Willey CJ, Blais JD, Hall AK, Krasa HB, Makin AJ, Czerwiec FS. Prevalence of autosomal dominant polycystic kidney disease in the European Union. *Nephrol Dial Transplant.* 2017;32(8). doi:10.1093/ndt/gfw240
7. Gaur P, Gedroyc W, Hill P. ADPKD-what the radiologist should know. *Br J Radiol.* 2019;92(1098). doi:10.1259/bjr.20190078
8. Ong ACM, Devuyst O, Knebelmann B, Walz G. Autosomal dominant polycystic kidney disease: The changing face of clinical management. *Lancet.* 2015;385(9981). doi:10.1016/S0140-6736(15)60907-2
9. Boucher C, Stanford R. Autosomal dominant polycystic kidney disease (ADPKD, MIM 173900, PKD1 and PKD2 genes, protein products known as polycystin-1 and polycystin-2). *Eur J Hum Genet.* 2004;12(5). doi:10.1038/sj.ejhg.5201162
10. Calvet JP. Ciliary signaling goes down the tubes. *Nat Genet.* 2003;33(2). doi:10.1038/ng1078
11. Torres VE, Harris PC, Pirson Y. Autosomal dominant polycystic kidney disease. *Lancet.* 2007;369(9569). doi:10.1016/S0140-6736(07)60601-1
12. Cornec-Le Gall E, Torres VE, Harris PC. Genetic complexity of autosomal dominant polycystic kidney and liver diseases. *J Am Soc Nephrol.* 2018;29(1). doi:10.1681/ASN.2017050483
13. Hughes J, Ward CJ, Peral B, et al. The polycystic kidney disease 1 (PKD1) gene encodes a novel protein with multiple cell recognition domains. *Nat Genet.* 1995;10(2). doi:10.1038/ng0695-151
14. Tan YC, Blumenfeld J, Rennert H. Autosomal dominant polycystic kidney disease: Genetics, mutations and microRNAs. *Biochim Biophys Acta - Mol Basis Dis.* 2011;1812(10). doi:10.1016/j.bbadis.2011.03.002
15. Ong ACM, Harris PC. Molecular pathogenesis of ADPKD: The polycystin complex gets complex. *Kidney Int.* 2005;67(4). doi:10.1111/j.1523-1755.2005.00201.x
16. Zhou J, Pei Y. Autosomal Dominant Polycystic Kidney Disease . In: *Molecular and Genetic Basis of Renal Disease.* ; 2003:88-117.
17. Cornec-Le Gall E, Audrézet MP, Chen JM, et al. Type of PKD1 mutation influences renal outcome in ADPKD. *J Am Soc Nephrol.* 2013;24(6). doi:10.1681/ASN.2012070650
18. Cornec-Le Gall E, Alam A, Perrone RD. Autosomal dominant polycystic kidney disease. *Lancet.* 2019;393(10174). doi:10.1016/S0140-6736(18)32782-X

19. Bergmann C, Guay-Woodford LM, Harris PC, Horie S, Peters DJM, Torres VE. Polycystic kidney disease. *Nat Rev Dis Prim.* 2018;4(1). doi:10.1038/s41572-018-0047-y
20. Vujic M, Heyer CM, Ars E, et al. Incompletely penetrant PKD1 alleles mimic the renal manifestations of ARPKD. *J Am Soc Nephrol.* 2010;21(7). doi:10.1681/ASN.2009101070
21. Pei Y, Lan Z, Wang K, et al. A missense mutation in PKD1 attenuates the severity of renal disease. *Kidney Int.* 2012;81(4). doi:10.1038/ki.2011.370
22. Heyer CM, Sundsbak JL, Abebe KZ, et al. Predicted mutation strength of nontruncating PKD1 mutations AIDS genotype-phenotype correlations in autosomal dominant polycystic kidney disease. *J Am Soc Nephrol.* 2016;27(9). doi:10.1681/ASN.2015050583
23. Porath B, Gainullin VG, Cornec-Le Gall E, et al. Mutations in GANAB , Encoding the Glucosidase II α Subunit, Cause Autosomal-Dominant Polycystic Kidney and Liver Disease. *Am J Hum Genet.* 2016;98(6). doi:10.1016/j.ajhg.2016.05.004
24. Cornec-Le Gall E, Olson RJ, Besse W, et al. Monoallelic Mutations to DNAJB11 Cause Atypical Autosomal-Dominant Polycystic Kidney Disease. *Am J Hum Genet.* 2018;102(5). doi:10.1016/j.ajhg.2018.03.013
25. Besse W, Dong K, Choi J, et al. Isolated polycystic liver disease genes define effectors of polycystin-1 function. *J Clin Invest.* 2017;127(5). doi:10.1172/JCI90129
26. Heidet L, Decramer S, Pawtowski A, et al. Spectrum of HNF1B mutations in a large cohort of patients who harbor renal diseases. *Clin J Am Soc Nephrol.* 2010;5(6). doi:10.2215/CJN.06810909
27. Cnossen WR, Te Morsche RHM, Hoischen A, et al. LRP5 variants may contribute to ADPKD. *Eur J Hum Genet.* 2016;24(2). doi:10.1038/ejhg.2015.86
28. Qian F, Germino FJ, Cai Y, Zhang X, Somlo S, Germino GG. PKD1 interacts with PKD2 through a probable coiled-coil domain. *Nat Genet.* 1997;16(2). doi:10.1038/ng0697-179
29. Forman JR, Qamar S, Paci E, Sandford RN, Clarke J. The remarkable mechanical strength of polycystin-1 supports a direct role in mechanotransduction. *J Mol Biol.* 2005;349(4). doi:10.1016/j.jmb.2005.04.008
30. Qian F, Wei W, Germino G, Oberhauser A. The nanomechanics of polycystin-1 extracellular region. *J Biol Chem.* 2005;280(49). doi:10.1074/jbc.M509650200
31. Xu M, Ma L, Bujalowski PJ, Qian F, Sutton RB, Oberhauser AF. Analysis of the REJ module of polycystin-1 using molecular modeling and force-spectroscopy techniques. *J Biophys.* Published online 2013. doi:10.1155/2013/525231
32. Torres VE, Harris PC. Autosomal dominant polycystic kidney disease: The last 3 years. *Kidney Int.* 2009;76(2). doi:10.1038/ki.2009.128
33. Qian F, Boletta A, Bhunia AK, et al. Cleavage of polycystin-1 requires the receptor for egg jelly domain and is disrupted by human autosomal-dominant polycystic kidney disease 1-associated mutations. *Proc Natl Acad Sci U S A.* 2002;99(26). doi:10.1073/pnas.252484899
34. Yu S, Hackmann K, Gao J, et al. Essential role of cleavage of Polycystin-1 at G protein-coupled receptor proteolytic site for kidney tubular structure. *Proc Natl Acad Sci.* 2007;104(47). doi:10.1073/pnas.0708217104
35. Gainullin VG, Hopp K, Ward CJ, Hommerding CJ, Harris PC. Polycystin-1 maturation requires polycystin-2 in a dose-dependent manner. *J Clin Invest.* 2015;125(2). doi:10.1172/JCI76972
36. Chapin HC, Rajendran V, Caplan MJ. Polycystin-1 surface localization is stimulated by polycystin-2 and cleavage at the g protein-coupled receptor proteolytic site. *Mol Biol Cell.*

2010;21(24). doi:10.1091/mbc.E10-05-0407

37. Shin Y Bin, Park JH. Recent Trends in ADPKD Research. In: Park J, Ahn C, eds. *Cystogenesis. Advances in Experimental Medicine and Biology*. Vol 933. Springer, Singapore; 2016. doi:10.1007/978-981-10-2041-4_1
38. Chauvet V, Tian X, Husson H, et al. Mechanical stimuli induce cleavage and nuclear translocation of the polycystin-1 C terminus. *J Clin Invest*. 2004;114(10). doi:10.1172/JCI21753
39. Low SH, Vasanth S, Larson CH, et al. Polycystin-1, STAT6, and P100 function in a pathway that transduces ciliary mechanosensation and is activated in polycystic kidney disease. *Dev Cell*. 2006;10(1). doi:10.1016/j.devcel.2005.12.005
40. Chapin HC, Caplan MJ. The cell biology of polycystic kidney disease. *J Cell Biol*. 2010;191(4). doi:10.1083/jcb.201006173
41. Scheffers MS. Polycystin-1, the product of the polycystic kidney disease 1 gene, co-localizes with desmosomes in MDCK cells. *Hum Mol Genet*. 2000;9(18). doi:10.1093/hmg/9.18.2743
42. Chauvet V, Qian F, Boute N, et al. Expression of PKD1 and PKD2 transcripts and proteins in human embryo and during normal kidney development. *Am J Pathol*. 2002;160(3). doi:10.1016/S0002-9440(10)64919-X
43. Su Q, Hu F, Ge X, et al. Structure of the human PKD1-PKD2 complex. *Science (80-)*. 2018;361(6406). doi:10.1126/science.aat9819
44. Cantiello HF, Montalbetti N, Li Q, Chen X. The Cytoskeletal Connection to Ion Channels as a Potential Mechanosensory Mechanism: Lessons from Polycystin-2 (TRPP2). In: *Current Topics in Membranes*. Vol 59. ; 2007. doi:10.1016/S1063-5823(06)59010-6
45. Montalbetti N, Li Q, Wu Y, Chen XZ, Cantiello HF. Polycystin-2 cation channel function in the human syncytiotrophoblast is regulated by microtubular structures. *J Physiol*. 2007;579(3). doi:10.1113/jphysiol.2006.125583
46. Markoff A, Bogdanova N, Knop M, et al. Annexin A5 Interacts with Polycystin-1 and Interferes with the Polycystin-1 Stimulated Recruitment of E-cadherin into Adherens Junctions. *J Mol Biol*. 2007;369(4). doi:10.1016/j.jmb.2007.03.070
47. Boca M, D'Amato L, Distefano G, Polishchuk RS, Germino GG, Boletta A. Polycystin-1 Induces Cell Migration by Regulating Phosphatidylinositol 3-kinase-dependent Cytoskeletal Rearrangements and GSK3 β -dependent Cell-Cell Mechanical Adhesion. *Mol Biol Cell*. 2007;18(10). doi:10.1091/mbc.e07-02-0142
48. Mochizuki T, Wu G, Hayashi T, et al. PKD2, a gene for polycystic kidney disease that encodes an integral membrane protein. *Science (80-)*. 1996;272(5266). doi:10.1126/science.272.5266.1339
49. Allen MD, Qamar S, Vadivelu MK, Sandford RN, Bycroft M. A high-resolution structure of the EF-hand domain of human polycystin-2. *Protein Sci*. 2014;23(9). doi:10.1002/pro.2513
50. Giamarchi A, Feng S, Rodat-Despoix L, et al. A polycystin-2 (TRPP2) dimerization domain essential for the function of heteromeric polycystin complexes. *EMBO J*. 2010;29(7). doi:10.1038/emboj.2010.18
51. Yu Y, Ulbrich MH, Li MH, et al. Structural and molecular basis of the assembly of the TRPP2/PKD1 complex. *Proc Natl Acad Sci U S A*. 2009;106(28). doi:10.1073/pnas.0903684106
52. Ong ACM, Ward CJ, Butler RJ, et al. Coordinate expression of the autosomal dominant polycystic kidney disease proteins, polycystin-2 and polycystin-1, in normal and cystic tissue. *Am J Pathol*. 1999;154(6). doi:10.1016/S0002-9440(10)65428-4

53. Torres VE, Cai Y, Chen X, et al. Vascular expression of polycystin-2. *J Am Soc Nephrol*. 2001;12(1). doi:10.1681/asn.v84616
54. Du J, Fu J, Xia XM, Shen B. The functions of TRPP2 in the vascular system. *Acta Pharmacol Sin*. 2016;37(1). doi:10.1038/aps.2015.126
55. Giamarchi A, Padilla F, Coste B, et al. The versatile nature of the calcium-permeable cation channel TRPP2. *EMBO Rep*. 2006;7(8). doi:10.1038/sj.embor.7400745
56. González-Perrett S, Kim K, Ibarra C, et al. Polycystin-2, the protein mutated in autosomal dominant polycystic kidney disease (ADPKD), is a Ca²⁺-permeable nonselective cation channel. *Proc Natl Acad Sci U S A*. 2001;98(3). doi:10.1073/pnas.98.3.1182
57. Koulen P, Cai Y, Geng L, et al. Polycystin-2 is an intracellular calcium release channel. *Nat Cell Biol*. 2002;4(3). doi:10.1038/ncb754
58. Grieben M, Pike ACW, Shintre CA, et al. Structure of the polycystic kidney disease TRP channel Polycystin-2 (PC2). *Nat Struct Mol Biol*. 2017;24(2). doi:10.1038/nsmb.3343
59. Abdi A, Mazzocco C, Légeron FP, Yvert B, Macrez N, Morel JL. TRPP2 modulates ryanodine- and inositol-1,4,5-trisphosphate receptors-dependent Ca²⁺ signals in opposite ways in cerebral arteries. *Cell Calcium*. 2015;58(5). doi:10.1016/j.ceca.2015.07.003
60. Chen XZ, Li Q, Wu Y, Liang G, Lara CJ, Cantiello HF. Submembraneous microtubule cytoskeleton: Interaction of TRPP2 with the cell cytoskeleton. *FEBS J*. 2008;275(19). doi:10.1111/j.1742-4658.2008.06616.x
61. Nauli SM, Alenghat FJ, Luo Y, et al. Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. *Nat Genet*. 2003;33(2). doi:10.1038/ng1076
62. Praetorius HA, Spring KR. Bending the MDCK cell primary cilium increases intracellular calcium. *J Membr Biol*. 2001;184(1). doi:10.1007/s00232-001-0075-4
63. Jin X, Mohieldin AM, Muntean BS, et al. Cilioplasm is a cellular compartment for calcium signaling in response to mechanical and chemical stimuli. *Cell Mol Life Sci*. 2014;71(11). doi:10.1007/s00018-013-1483-1
64. Xu C, Rossetti S, Jiang L, et al. Human ADPKD primary cyst epithelial cells with a novel, single codon deletion in the PKD1 gene exhibit defective ciliary polycystin localization and loss of flow-induced Ca²⁺ signaling. *Am J Physiol - Ren Physiol*. 2007;292(3). doi:10.1152/ajprenal.00285.2006
65. Spirli C, Locatelli L, Fiorotto R, et al. Altered store operated calcium entry increases cyclic 3',5'-adenosine monophosphate production and extracellular signal-regulated kinases 1 and 2 phosphorylation in polycystin-2-defective cholangiocytes. *Hepatology*. 2012;55(3). doi:10.1002/hep.24723
66. Yamaguchi T, Hempson SJ, Reif GA, Hedge AM, Wallace DP. Calcium restores a normal proliferation phenotype in human polycystic kidney disease epithelial cells. *J Am Soc Nephrol*. 2006;17(1). doi:10.1681/ASN.2005060645
67. Delling M, Indzhukulian AA, Liu X, et al. Primary cilia are not calcium-responsive mechanosensors. *Nature*. 2016;531(7596). doi:10.1038/nature17426
68. van de Laarschot LFM, te Morsche RHM, Hoischen A, et al. Novel GANAB variants associated with polycystic liver disease. *Orphanet J Rare Dis*. 2020;15(1). doi:10.1186/s13023-020-01585-4
69. Pelletier MF, Marcil A, Sevigny G, et al. The heterodimeric structure of glucosidase II is required for its activity, solubility, and localization in vivo. *Glycobiology*. 2000;10(8). doi:10.1093/glycob/10.8.815

70. GeneCards - The human gene database. GANAB Gene - Glucosidase II Alpha Subunit. GeneCards. <https://www.genecards.org/cgi-bin/carddisp.pl?gene=GANAB#>
71. Yu M, Haslam RHA, Haslam DB. HEDJ, an Hsp40 co-chaperone localized to the endoplasmic reticulum of human cells. *J Biol Chem*. 2000;275(32). doi:10.1074/jbc.M000739200
72. Shen Y, Hendershot LM. ERdj3, a stress-inducible endoplasmic reticulum DnaJ homologue, serves as a cofactor for BiP's interactions with unfolded substrates. *Mol Biol Cell*. 2005;16(1). doi:10.1091/mbc.E04-05-0434
73. Allison SJ. DNAJB11: Another player in ADPKD. *Nat Rev Nephrol*. 2018;14(8). doi:10.1038/s41581-018-0024-4
74. Krappitz M, Gallagher AR, Fedeles S. Is it Time to Fold the Cysts Away? *Trends Mol Med*. 2016;22(12). doi:10.1016/j.molmed.2016.10.001
75. Paul BM, Vanden Heuvel GB. Kidney: polycystic kidney disease. *Wiley Interdiscip Rev Dev Biol*. 2014;3(6). doi:10.1002/wdev.152
76. Reeders ST. Multilocus polycystic disease. *Nat Genet*. 1992;1(4). doi:10.1038/ng0792-235
77. Happé H, Peters DJM. Translational research in ADPKD: Lessons from animal models. *Nat Rev Nephrol*. 2014;10(10). doi:10.1038/nrneph.2014.137
78. Qian F, Watnick TJ, Onuchic LF, Germino GG. The molecular basis of focal cyst formation in human autosomal dominant polycystic kidney disease type I. *Cell*. 1996;87(6). doi:10.1016/S0092-8674(00)81793-6
79. Pei Y, Watnick T, He N, et al. Somatic PKD2 mutations in individual kidney and liver cysts support a "two-hit" model of cystogenesis in type 2 autosomal dominant polycystic kidney disease. *J Am Soc Nephrol*. 1999;10(7). doi:10.1681/asn.v1071524
80. Watnick T, He N, Wang K, et al. Mutations of PKD1 in ADPKD2 cysts suggest a pathogenic effect of trans- heterozygous mutations. *Nat Genet*. 2000;25(2). doi:10.1038/75981
81. Brasier JL, Henske EP. Loss of the polycystic kidney disease (PKD1) region of chromosome 16p13 in renal cyst cells supports a loss-of-function model for cyst pathogenesis. *J Clin Invest*. 1997;99(2). doi:10.1172/JCI119147
82. Torra R, Badenas C, San Millán JL, Pérez-Oller L, Estivill X, Darnell A. A loss-of-function model for cystogenesis in human autosomal dominant polycystic kidney disease type 2. *Am J Hum Genet*. 1999;65(2). doi:10.1086/302501
83. Wu G, D'Agati V, Cai Y, et al. Somatic inactivation of Pkd2 results in polycystic kidney disease. *Cell*. 1998;93(2). doi:10.1016/S0092-8674(00)81570-6
84. Lantinga-van Leeuwen IS, Dauwerse JG, Baelde HJ, et al. Lowering of Pkd1 expression is sufficient to cause polycystic kidney disease. *Hum Mol Genet*. 2004;13(24). doi:10.1093/hmg/ddh336
85. Jiang ST, Chiou YY, Wang E, et al. Defining a link with autosomal-dominant polycystic kidney disease in mice with congenitally low expression of Pkd1. *Am J Pathol*. 2006;168(1). doi:10.2353/ajpath.2006.050342
86. Rossetti S, Kubly VJ, Consugar MB, et al. Incompletely penetrant PKD1 alleles suggest a role for gene dosage in cyst initiation in polycystic kidney disease. *Kidney Int*. 2009;75(8). doi:10.1038/ki.2008.686
87. Gallagher AR, Germino GG, Somlo S. Molecular Advances in Autosomal Dominant Polycystic Kidney Disease. *Adv Chronic Kidney Dis*. 2010;17(2). doi:10.1053/j.ackd.2010.01.002

88. Harris PC. What is the role of somatic mutation in autosomal dominant polycystic kidney disease? *J Am Soc Nephrol.* 2010;21(7). doi:10.1681/ASN.2010030328
89. Hopp K, Ward CJ, Hommerding CJ, et al. Functional polycystin-1 dosage governs autosomal dominant polycystic kidney disease severity. *J Clin Invest.* 2012;122(11). doi:10.1172/JCI64313
90. Lantinga-van leeuwen IS, Leonhard WN, van der Wal A, Breuning MH, de Heer E, Peters DJM. Kidney-specific inactivation of the Pkd1 gene induces rapid cyst formation in developing kidneys and a slow onset of disease in adult mice. *Hum Mol Genet.* 2007;16(24). doi:10.1093/hmg/ddm299
91. Saigusa T, Darwin Bell P. Molecular pathways and therapies in autosomal-dominant polycystic kidney disease. *Physiology.* 2015;30(3). doi:10.1152/physiol.00032.2014
92. Lugnier C. Cyclic nucleotide phosphodiesterase (PDE) superfamily: A new target for the development of specific therapeutic agents. *Pharmacol Ther.* 2006;109(3). doi:10.1016/j.pharmthera.2005.07.003
93. Yamaguchi T, Nagao S, Kasahara M, Takahashi H, Grantham JJ. Renal accumulation and excretion of cyclic adenosine monophosphate in a murine model of slowly progressive polycystic kidney disease. *Am J Kidney Dis.* 1997;30(5). doi:10.1016/S0272-6386(97)90496-0
94. Wallace DP. Cyclic AMP-mediated cyst expansion. *Biochim Biophys Acta - Mol Basis Dis.* 2011;1812(10). doi:10.1016/j.bbadis.2010.11.005
95. Hanaoka K, Guggino WB. cAMP regulates cell proliferation and cyst formation in autosomal polycystic kidney disease cells. *J Am Soc Nephrol.* 2000;11(7). doi:10.1681/asn.v1171179
96. Lee SH, Somlo S. Cyst growth, polycystins, and primary cilia in autosomal dominant polycystic kidney disease. *Kidney Res Clin Pract.* 2014;33(2). doi:10.1016/j.krcp.2014.05.002
97. Yamaguchi T, Nagao S, Wallace DP, et al. Cyclic AMP activates B-Raf and ERK in cyst epithelial cells from autosomal-dominant polycystic kidneys. *Kidney Int.* 2003;63(6). doi:10.1046/j.1523-1755.2003.00023.x
98. Chebib FT, Sussman CR, Wang X, Harris PC, Torres VE. Vasopressin and disruption of calcium signalling in polycystic kidney disease. *Nat Rev Nephrol.* 2015;11(8). doi:10.1038/nrneph.2015.39
99. Rees S, Kittikulsuth W, Roos K, Strait KA, Van Hoek A, Kohan DE. Adenylyl cyclase 6 deficiency ameliorates polycystic kidney disease. *J Am Soc Nephrol.* 2014;25(2). doi:10.1681/ASN.2013010077
100. Van Gastel MDA, Torres VE. Polycystic Kidney Disease and the Vasopressin Pathway. *Ann Nutr Metab.* 2017;70(Suppl1). doi:10.1159/000463063
101. Zittema D, Boertien WE, van Beek AP, et al. Vasopressin, copeptin, and renal concentrating capacity in patients with autosomal dominant polycystic kidney disease without renal impairment. *Clin J Am Soc Nephrol.* 2012;7(6). doi:10.2215/CJN.11311111
102. Bankir L, Bouby N, Ritz E. Vasopressin: A novel target for the prevention and retardation of kidney disease? *Nat Rev Nephrol.* 2013;9(4). doi:10.1038/nrneph.2013.22
103. Morgenthaler NG. Copeptin: A biomarker of cardiovascular and renal function. *Congest Hear Fail.* 2010;16(SUPPL. 1). doi:10.1111/j.1751-7133.2010.00177.x
104. Torres VE. Vasopressin receptor antagonists, heart failure, and polycystic kidney disease. *Annu Rev Med.* 2015;66. doi:10.1146/annurev-med-050913-022838
105. Devuyst O, Chapman AB, Shoaf SE, Czerwiec FS, Blais JD. Tolerability of Aquaretic-Related Symptoms Following Tolvaptan for Autosomal Dominant Polycystic Kidney Disease: Results

From TEMPO 3:4. *Kidney Int Reports*. 2017;2(6). doi:10.1016/j.ekir.2017.07.004

106. Horie S. Will introduction of tolvaptan change clinical practice in autosomal dominant polycystic kidney disease? *Kidney Int*. 2015;88(1). doi:10.1038/ki.2015.143
107. Amaral MD, Hutt DM, Tomati V, Botelho HM, Pedemonte N. CFTR processing, trafficking and interactions. *J Cyst Fibros*. 2020;19. doi:10.1016/j.jcf.2019.10.017
108. Saint-Criq V, Gray MA. Role of CFTR in epithelial physiology. *Cell Mol Life Sci*. 2017;74(1). doi:10.1007/s00018-016-2391-y
109. Hanaoka K, Devuyst O, Schwiebert EM, Wilson PD, Guggino WB. A role for CFTR in human autosomal dominant polycystic kidney disease. *Am J Physiol - Cell Physiol*. 1996;270(1 39-1). doi:10.1152/ajpcell.1996.270.1.c389
110. Bagnat M, Navis A, Marjoram L. Cftr controls lumen expansion and function of Kupffer's vesicle in zebrafish. *Dev*. 2013;140(8). doi:10.1242/dev.091819
111. Zoncu R, Efeyan A, Sabatini DM. mTOR: From growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol*. 2011;12(1). doi:10.1038/nrm3025
112. Wullschlegel S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell*. 2006;124(3). doi:10.1016/j.cell.2006.01.016
113. Becker JU, Opazo Saez A, Zerres K, et al. The mTOR pathway is activated in human autosomal-recessive polycystic kidney disease. *Kidney Blood Press Res*. 2010;33(2). doi:10.1159/000314380
114. Dere R, Wilson PD, Sandford RN, Walker CL. Carboxy terminal tail of polycystin-1 regulates localization of TSC2 to repress mTOR. *PLoS One*. 2010;5(2). doi:10.1371/journal.pone.0009239
115. Serra AL, Poster D, Kistler AD, et al. Sirolimus and Kidney Growth in Autosomal Dominant Polycystic Kidney Disease. *N Engl J Med*. 2010;363(9). doi:10.1056/nejmoa0907419
116. Wu M, Wahl PR, Le Hir M, Wäckerle-Men Y, Wüthrich RP, Serra AL. Everolimus retards cyst growth and preserves kidney function in a rodent model for polycystic kidney disease. *Kidney Blood Press Res*. 2007;30(4). doi:10.1159/000104818
117. Lal M, Song X, Pluznick JL, et al. Polycystin-1 C-terminal tail associates with β -catenin and inhibits canonical Wnt signaling. *Hum Mol Genet*. 2008;17(20). doi:10.1093/hmg/ddn208
118. Grantham JJ. Autosomal Dominant Polycystic Kidney Disease. *N Engl J Med*. 2008;359(14). doi:10.1056/NEJMc0804458
119. Nichols MT, Gidey E, Matzakos T, et al. Secretion of cytokines and growth factors into autosomal dominant polycystic kidney disease liver cyst fluid. *Hepatology*. 2004;40(4). doi:10.1002/hep.1840400413
120. Fabris L, Cadamuro M, Fiorotto R, et al. Effects of angiogenic factor overexpression by human and rodent cholangiocytes in polycystic liver diseases. *Hepatology*. 2006;43(5). doi:10.1002/hep.21143
121. Bae KT, Zhu F, Chapman AB, et al. Magnetic resonance imaging evaluation of hepatic cysts in early autosomal-dominant polycystic kidney disease: the Consortium for Radiologic Imaging Studies of Polycystic Kidney Disease cohort. *Clin J Am Soc Nephrol*. 2006;1(1). doi:10.2215/CJN.00080605
122. Lederman ED, McCoy G, Conti DJ, Lee EC. Diverticulitis and polycystic kidney disease. In: *American Surgeon*. Vol 66. ; 2000.
123. Chapman AB, Guay-Woodford LM, Grantham JJ, et al. Renal structure in early autosomal-

- dominant polycystic kidney disease (ADPKD): The Consortium for Radiologic Imaging Studies of Polycystic Kidney Disease (CRISP) cohort. *Kidney Int.* 2003;64(3). doi:10.1046/j.1523-1755.2003.00185.x
124. Chapman AB, Stepniakowski K, Rahbari-Oskoui F. Hypertension in Autosomal Dominant Polycystic Kidney Disease. *Adv Chronic Kidney Dis.* 2010;17(2). doi:10.1053/j.ackd.2010.01.001
 125. Seeman T, Dušek J, Vondřichová H, et al. Ambulatory blood pressure correlates with renal volume and number of renal cysts in children with autosomal dominant polycystic kidney disease. *Blood Press Monit.* 2003;8(3). doi:10.1097/00126097-200306000-00003
 126. Marlais M, Cuthell O, Langan D, Dudley J, Sinha MD, Winyard PJD. Hypertension in autosomal dominant polycystic kidney disease: A meta-analysis. *Arch Dis Child.* 2016;101(12). doi:10.1136/archdischild-2015-310221
 127. Cadnapaphornchai MA, McFann K, Strain JD, Masoumi A, Schrier RW. Increased left ventricular mass in children with autosomal dominant polycystic kidney disease and borderline hypertension. *Kidney Int.* 2008;74(9). doi:10.1038/ki.2008.397
 128. Fick GM, Johnson AM, Hammond WS, Gabow PA. Causes of death in autosomal dominant polycystic kidney disease. *J Am Soc Nephrol.* 1995;5(12). doi:10.1681/asn.v5i122048
 129. Nehme A, Zouein FA, Zayeri ZD, Zibara K. An Update on the Tissue Renin Angiotensin System and Its Role in Physiology and Pathology. *J Cardiovasc Dev Dis.* 2019;6(2). doi:10.3390/jcdd6020014
 130. Fountain JH, Lappin SL. Physiology, Renin Angiotensin System - StatPearls - NCBI Bookshelf. NCBI.
 131. Beierwaltes WH. The role of calcium in the regulation of renin secretion. *Am J Physiol - Ren Physiol.* 2010;298(1). doi:10.1152/ajprenal.00143.2009
 132. Loghman-Adham M, Soto CE, Inagami T, Cassis L. The intrarenal renin-angiotensin system in autosomal dominant polycystic kidney disease. *Am J Physiol - Ren Physiol.* 2004;287(4 56-4). doi:10.1152/ajprenal.00370.2003
 133. Nauli SM, Kawanabe Y, Kaminski JJ, Pearce WJ, Ingber DE, Zhou J. Endothelial cilia are fluid shear sensors that regulate calcium signaling and nitric oxide production through polycystin-1. *Circulation.* 2008;117(9). doi:10.1161/CIRCULATIONAHA.107.710111
 134. Jones TJ, Adapala RK, Geldenhuys WJ, et al. Primary cilia regulates the directional migration and barrier integrity of endothelial cells through the modulation of Hsp27 dependent actin cytoskeletal organization. *J Cell Physiol.* 2012;227(1). doi:10.1002/jcp.22704
 135. Aboualaiwi WA, Takahashi M, Mell BR, et al. Ciliary polycystin-2 is a mechanosensitive calcium channel involved in nitric oxide signaling cascades. *Circ Res.* 2009;104(7). doi:10.1161/CIRCRESAHA.108.192765
 136. Qian Q, Hunter LW, Li M, et al. Pkd2 haploinsufficiency alters intracellular calcium regulation in vascular smooth muscle cells. *Hum Mol Genet.* 2003;12(15). doi:10.1093/hmg/ddg190
 137. Narayanan D, Bulley S, Leo MD, et al. Smooth muscle cell transient receptor potential polycystin-2 (TRPP2) channels contribute to the myogenic response in cerebral arteries. *J Physiol.* 2013;591(20). doi:10.1113/jphysiol.2013.258319
 138. Bulley S, Fernández-Peña C, Hasan R, et al. Arterial smooth muscle cell PKD2 (TRPP1) channels regulate systemic blood pressure. *Elife.* 2018;7. doi:10.7554/eLife.42628
 139. Kuo IY, Chapman AB. Polycystins, ADPKD, and Cardiovascular Disease. *Kidney Int Reports.* 2020;5(4). doi:10.1016/j.ekir.2019.12.007

140. Cagnazzo F, Gambacciani C, Morganti R, Perrini P. Intracranial aneurysms in patients with autosomal dominant polycystic kidney disease: prevalence, risk of rupture, and management. A systematic review. *Acta Neurochir (Wien)*. 2017;159(5). doi:10.1007/s00701-017-3142-z
141. Hussain S. Intracranial Aneurysm Development 101: Pathogenesis and Risk Factors. Consult QD. Published 2019. <https://consultqd.clevelandclinic.org/intracranial-aneurysm-development-101-pathogenesis-and-risk-factors/>
142. Kuo IY, Chapman A. Intracranial Aneurysms in ADPKD. *Clin J Am Soc Nephrol*. 2019;14(8). doi:10.2215/cjn.07570719
143. Krishnappa V, Vinod P, Deverakonda D, Raina R. Autosomal dominant polycystic kidney disease and the heart and brain. *Cleve Clin J Med*. 2017;84(6). doi:10.3949/ccjm.84a.16107
144. Pirson Y. Extrarenal Manifestations of Autosomal Dominant Polycystic Kidney Disease. *Adv Chronic Kidney Dis*. 2010;17(2). doi:10.1053/j.ackd.2010.01.003
145. Drummond IA. Polycystins, focal adhesions and extracellular matrix interactions. *Biochim Biophys Acta - Mol Basis Dis*. 2011;1812(10). doi:10.1016/j.bbadis.2011.03.003
146. Kip SN, Hunter LW, Ren Q, et al. [Ca²⁺]_i reduction increases cellular proliferation and apoptosis in vascular smooth muscle cells relevance to the ADPKD phenotype. *Circ Res*. 2005;96(8). doi:10.1161/01.RES.0000163278.68142.8a
147. Leier C V., Baker PB, Kilman JW, Wooley CF. Cardiovascular abnormalities associated with adult polycystic kidney disease. *Ann Intern Med*. 1984;100(5). doi:10.7326/0003-4819-100-5-683
148. Lumiaho A, Ikäheimo R, Miettinen R, et al. Mitral valve prolapse and mitral regurgitation are common in patients with polycystic kidney disease type 1. *Am J Kidney Dis*. 2001;38(6). doi:10.1053/ajkd.2001.29216
149. Koren MJ, Devereux RB, Casale PN, Savage DD, Laragh JH. Relation of left ventricular mass and geometry to morbidity and mortality in uncomplicated essential hypertension. *Ann Intern Med*. 1991;114(5). doi:10.7326/0003-4819-114-5-345
150. Gabow PA, Johnson AM, Kaehny WD, et al. Factors affecting the progression of renal disease in autosomal-dominant polycystic kidney disease. *Kidney Int*. 1992;41(5). doi:10.1038/ki.1992.195
151. Saggar-Malik AK, Missouris CG, Gill JS, Singer DRJ, Markandu ND, Macgregor GA. Left ventricular mass in normotensive subjects with autosomal dominant polycystic kidney disease. *BMJ*. 1994;309(6969). doi:10.1136/bmj.309.6969.1617
152. Wu G, Markowitz GS, Li L, et al. Cardiac defects and renal failure in mice with targeted mutations in Pkd2. *Nat Genet*. 2000;24(1). doi:10.1038/71724
153. Boulter C, Mulroy S, Webb S, Fleming S, Brindle K, Sandford R. Cardiovascular, skeletal, and renal defects in mice with a targeted disruption of the Pkd1 gene. *Proc Natl Acad Sci U S A*. 2001;98(21). doi:10.1073/pnas.211191098
154. Prasad H, Dang DK, Kondapalli KC, Natarajan N, Cebotaru V, Rao R. NHA2 promotes cyst development in an in vitro model of polycystic kidney disease. *J Physiol*. 2019;597(2). doi:10.1113/JP276796
155. Seoung WC, Cho EY, Moon SP, Lee KB, Kim H, Kim U. Polycystin-1 expression in fetal, adult and autosomal dominant polycystic kidney. *J Korean Med Sci*. 2006;21(3). doi:10.3346/jkms.2006.21.3.425
156. Doctor RB, Serkova NJ, Hasebroock KM, Zafar I, Edelstein CL. Distinct patterns of kidney and liver cyst growth in pkd2(WS25/-) mice. *Nephrol Dial Transplant*. 2010;25(11).

doi:10.1093/ndt/gfq195

157. Elberg G, Elberg D, Lewis T V., et al. EP2 receptor mediates PGE2-induced cystogenesis of human renal epithelial cells. *Am J Physiol - Ren Physiol.* 2007;293(5). doi:10.1152/ajprenal.00036.2007
158. Happé H, Leonhard WN, van der Wal A, et al. Toxic tubular injury in kidneys from Pkd1-deletion mice accelerates cystogenesis accompanied by dysregulated planar cell polarity and canonical Wnt signaling pathways. *Hum Mol Genet.* 2009;18(14). doi:10.1093/hmg/ddp190
159. Leonhard WN, van der Wal A, Novalic Z, et al. Curcumin inhibits cystogenesis by simultaneous interference of multiple signaling pathways: In vivo evidence from a Pkd1-deletion model. *Am J Physiol - Ren Physiol.* 2011;300(5). doi:10.1152/ajprenal.00419.2010
160. Luyten A, Su X, Gondela S, et al. Aberrant regulation of planar cell polarity in polycystic kidney disease. *J Am Soc Nephrol.* 2010;21(9). doi:10.1681/ASN.2010010127
161. Aguiari G, Varani K, Bogo M, et al. Deficiency of polycystic kidney disease-1 gene (PKD1) expression increases A3 adenosine receptors in human renal cells: Implications for cAMP-dependent signalling and proliferation of PKD1-mutated cystic cells. *Biochim Biophys Acta - Mol Basis Dis.* 2009;1792(6). doi:10.1016/j.bbadis.2009.03.001
162. Gattone VH, Wang X, Harris PC, Torres VE. Inhibition of renal cystic disease development and progression by a vasopressin V2 receptor antagonist. *Nat Med.* 2003;9(10). doi:10.1038/nm935
163. Yuajit C, Homvisasevongsa S, Chatsudthipong L, Soodvilai S, Muanprasat C, Chatsudthipong V. Steviol Reduces MDCK Cyst Formation and Growth by Inhibiting CFTR Channel Activity and Promoting Proteasome-Mediated CFTR Degradation. *PLoS One.* 2013;8(3). doi:10.1371/journal.pone.0058871
164. Bakkers J. Zebrafish as a model to study cardiac development and human cardiac disease. *Cardiovasc Res.* 2011;91(2). doi:10.1093/cvr/cvr098
165. Gore A V., Monzo K, Cha YR, Pan W, Weinstein BM. Vascular development in the zebrafish. *Cold Spring Harb Perspect Med.* 2012;2(5). doi:10.1101/cshperspect.a006684
166. Lieschke GJ, Currie PD. Animal models of human disease: Zebrafish swim into view. *Nat Rev Genet.* 2007;8(5). doi:10.1038/nrg2091
167. Hill AJ, Teraoka H, Heideman W, Peterson RE. Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicol Sci.* 2005;86(1). doi:10.1093/toxsci/kfi110
168. Karlsson J, Von Hofsten J, Olsson PE. Generating transparent zebrafish: A refined method to improve detection of gene expression during embryonic development. *Mar Biotechnol.* 2001;3(6). doi:10.1007/s1012601-0053-4
169. Postlethwait JH, Yan YL, Gates MA, et al. Vertebrate genome evolution and the zebrafish gene map. *Nat Genet.* 1998;18(4). doi:10.1038/ng0498-345
170. Postlethwait JH, Woods IG, Ngo-Hazelett P, et al. Zebrafish comparative genomics and the origins of vertebrate chromosomes. *Genome Res.* 2000;10(12). doi:10.1101/gr.164800
171. England SJ, Campbell PC, Banerjee S, Swanson AJ, Lewis KE. Identification and Expression Analysis of the Complete Family of Zebrafish pkd Genes. *Front Cell Dev Biol.* 2017;5. doi:10.3389/fcell.2017.00005
172. Schottenfeld J, Sullivan-Brown J, Burdine RD. Zebrafish curly up encodes a Pkd2 ortholog that restricts left-side-specific expression of southpaw. *Development.* 2007;134(8). doi:10.1242/dev.02827
173. Obara T, Mangos S, Liu Y, et al. Polycystin-2 immunolocalization and function in zebrafish. *J*

Am Soc Nephrol. 2006;17(10). doi:10.1681/ASN.2006040412

174. Roxo-Rosa M, Jacinto R, Sampaio P, Lopes SS. The zebrafish Kupffer's vesicle as a model system for the molecular mechanisms by which the lack of Polycystin-2 leads to stimulation of CFTR. *Biol Open.* 2015;4(11). doi:10.1242/bio.014076
175. Sampaio P, Ferreira RR, Guerrero A, et al. Left-right organizer flow dynamics: How much cilia activity reliably yields laterality? *Dev Cell.* 2014;29(6). doi:10.1016/j.devcel.2014.04.030
176. Metzner A, Griffiths JD, Streets AJ, et al. A high throughput zebrafish chemical screen reveals ALK5 and non-canonical androgen signalling as modulators of the *pkd2*^{-/-} phenotype. *Sci Rep.* 2020;10(1). doi:10.1038/s41598-019-56995-7
177. Gaj T, Sirk SJ, Shui SL, Liu J. Genome-editing technologies: Principles and applications. *Cold Spring Harb Perspect Biol.* 2016;8(12). doi:10.1101/cshperspect.a023754
178. Paavola J, Schliffke S, Rossetti S, et al. Polycystin-2 mutations lead to impaired calcium cycling in the heart and predispose to dilated cardiomyopathy. *J Mol Cell Cardiol.* 2013;58(1). doi:10.1016/j.yjmcc.2013.01.015
179. Bill BR, Petzold AM, Clark KJ, Schimmenti LA, Ekker SC. A primer for morpholino use in zebrafish. *Zebrafish.* 2009;6(1). doi:10.1089/zeb.2008.0555
180. Kok FO, Shin M, Ni CW, et al. Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. *Dev Cell.* 2015;32(1). doi:10.1016/j.devcel.2014.11.018
181. Rossi A, Kontarakis Z, Gerri C, et al. Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature.* 2015;524(7564). doi:10.1038/nature14580
182. Timme-Laragy AR, Karchner SI, Hahn ME. Gene knockdown by morpholino-modified oligonucleotides in the zebrafish (*danio rerio*) model: Applications for developmental toxicology. *Methods Mol Biol.* 2012;889. doi:10.1007/978-1-61779-867-2_5
183. Morcos PA, Vincent AC, Moulton JD. Gene editing versus morphants. *Zebrafish.* 2015;12(5). doi:10.1089/zeb.2015.1114
184. Qian Q, Hunter LW, Du H, Ren Q, Han Y, Sieck GC. *Pkd2*^{+/-} vascular smooth muscles develop exaggerated vasocontraction in response to phenylephrine stimulation. *J Am Soc Nephrol.* 2007;18(2). doi:10.1681/ASN.2006050501
185. Galloway JL, Wingert RA, Thisse C, Thisse B, Zon LI. Loss of Gata1 but not Gata2 converts erythropoiesis to myelopoiesis in zebrafish embryos. *Dev Cell.* 2005;8(1). doi:10.1016/j.devcel.2004.12.001
186. Brown LA, Rodaway ARF, Schilling TF, et al. Insights into early vasculogenesis revealed by expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos. *Mech Dev.* 2000;90(2). doi:10.1016/S0925-4773(99)00256-7
187. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn.* 1995;203(3). doi:10.1002/aja.1002030302
188. Xin Y, Duan C. Microinjection of antisense morpholinos, CRISPR/Cas9 RNP, and RNA/DNA into zebrafish embryos. In: *Methods in Molecular Biology*. Vol 1742. ; 2018. doi:10.1007/978-1-4939-7665-2_18
189. Pei W, Burgess SM. Microinjection in zebrafish for genome editing and functional studies. In: *Methods in Molecular Biology*. Vol 1874. ; 2019. doi:10.1007/978-1-4939-8831-0_26
190. Zhang Y, Wiest DL. Using the zebrafish model to study T cell development. In: *T-Cell Development: Methods and Protocols*. ; 2015. doi:10.1007/978-1-4939-2809-5_22

191. Smith D, Montenegro-Johnson T, Lopes S. Organized chaos in Kupffer's vesicle: How a heterogeneous structure achieves consistent left-right patterning. *Bioarchitecture*. 2014;4(3). doi:10.4161/19490992.2014.956593
192. Oliveira I, Jacinto R, Pestana S, et al. Zebrafish Model as a Screen to Prevent Cyst Inflation in Autosomal Dominant Polycystic Kidney Disease. *Int J Mol Sci*. 2021;22(16). doi:10.3390/ijms22169013
193. Chen Q, Jiang L, Li C, et al. Haemodynamics-Driven Developmental Pruning of Brain Vasculature in Zebrafish. *PLoS Biol*. 2012;10(8). doi:10.1371/journal.pbio.1001374
194. Victoria AS, Roy B, Victoria LH. Development of an ImageJ-based method for analysing the developing zebrafish vasculature. *Vasc Cell*. 2017;9(1). doi:10.24238/13221-9-1-172
195. Weinstein BM, Stemple DL, Driever W, Fishman MC. Gridlock, a localized heritable vascular patterning defect in the zebrafish. *Nat Med*. 1995;1(11). doi:10.1038/nm1195-1143
196. Walcott BP, Peterson RT. Zebrafish models of cerebrovascular disease. *J Cereb Blood Flow Metab*. 2014;34(4). doi:10.1038/jcbfm.2014.27
197. Lawson ND, Weinstein BM. In Vivo Imaging of Embryonic Vascular Development Using Transgenic Zebrafish. *Dev Biol*. 2002;248(2). doi:10.1006/dbio.2002.0711
198. Rajesh Y, Biswas A, Banik P, et al. Transcriptional regulation of HSPB1 by Friend leukemia integration-1 factor modulates radiation and temozolomide resistance in glioblastoma. *Oncotarget*. 2020;11(13). doi:10.18632/oncotarget.27425
199. Mager AM, Grapin-Botton A, Ladjali K, et al. The avian fli gene is specifically expressed during embryogenesis in a subset of neural crest cells giving rise to mesenchyme. *Int J Dev Biol*. 1998;42(4). doi:10.1387/ijdb.9694627
200. Thompson MA, Ransom DG, Pratt SJ, et al. The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. *Dev Biol*. 1998;197(2). doi:10.1006/dbio.1998.8887
201. Isogai S, Horiguchi M, Weinstein BM. The Vascular Anatomy of the Developing Zebrafish: An Atlas of Embryonic and Early Larval Development. *Dev Biol*. 2001;230(2). doi:10.1006/dbio.2000.9995
202. Drummond I, Austin-Tse C. Zebrafish Cilia. In: ; 2013. doi:10.1016/B978-0-12-397944-5.00011-0
203. Mangos S, Lam PY, Zhao A, et al. The ADPKD genes pkd1a/b and pkd2 regulate extracellular matrix formation. *DMM Dis Model Mech*. 2010;3(5-6). doi:10.1242/dmm.003194
204. Isogai S, Lawson ND, Torrealday S, Horiguchi M, Weinstein BM. Angiogenic network formation in the developing vertebrate trunk. *Development*. 2003;130(21). doi:10.1242/dev.00733
205. Jacinto R, Sampaio P, Roxo-Rosa M, Pestana S, Lopes SS. Pkd2 Affects Cilia Length and Impacts LR Flow Dynamics and Dand5. *Front Cell Dev Biol*. 2021;9. doi:10.3389/fcell.2021.624531
206. Chen J. Impaired cardiovascular function caused by different stressors elicits a common pathological and transcriptional response in zebrafish embryos. *Zebrafish*. 2013;10(3). doi:10.1089/zeb.2013.0875
207. Kaufman R, Weiss O, Sebbagh M, et al. Development and origins of Zebrafish ocular vasculature. *BMC Dev Biol*. 2015;15(1). doi:10.1186/s12861-015-0066-9
208. Harrington ML, Rodney SB, Michael PM. Hydrocephalus. *Vet Clin North Am Small Anim Pract*. 1996;26(4). doi:10.1016/S0195-5616(96)50108-7

209. Kissa K, Herbolme P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature*. 2010;464(7285). doi:10.1038/nature08761
210. Sung PH, Yang YH, Chiang HJ, et al. Risk of aortic aneurysm and dissection in patients with autosomal-dominant polycystic kidney disease: A nationwide population-based cohort study. *Oncotarget*. 2017;8(34). doi:10.18632/oncotarget.16338
211. Field HA, Ober EA, Roeser T, Stainier DYR. Formation of the digestive system in zebrafish. I. Liver morphogenesis. *Dev Biol*. 2003;253(2). doi:10.1016/S0012-1606(02)00017-9
212. Eisen JS, Smith JC. Controlling morpholino experiments: Don't stop making antisense. *Development*. 2008;135(10). doi:10.1242/dev.001115
213. Sun Z, Amsterdam A, Pazour GJ, Cole DG, Miller MS, Hopkins N. A genetic screen in zebrafish identifies cilia genes as a principal cause of cystic kidney. *Development*. 2004;131(16). doi:10.1242/dev.01240
214. Sugden WW, Meissner R, Aegerter-Wilmsen T, et al. Endoglin controls blood vessel diameter through endothelial cell shape changes in response to haemodynamic cues. *Nat Cell Biol*. 2017;19(6). doi:10.1038/ncb3528
215. Du J, Ma X, Shen B, Huang Y, Birnbaumer L, Yao X. TRPV4, TRPC1, and TRPP2 assemble to form a flow-sensitive heteromeric channel. *FASEB J*. 2014;28(11). doi:10.1096/fj.14-251652
216. Zhao R, Zhou M, Li J, et al. Increased TRPP2 expression in vascular smooth muscle cells from high-salt intake hypertensive rats: The crucial role in vascular dysfunction. *Mol Nutr Food Res*. 2015;59(2). doi:10.1002/mnfr.201400465
217. Kulkeaw K, Sugiyama D. Zebrafish erythropoiesis and the utility of fish as models of anemia. *Stem Cell Res Ther*. 2012;3(6). doi:10.1186/scrt146
218. Xie X, Zhou T, Wang Y, et al. Blood Flow Regulates Zebrafish Caudal Vein Plexus Angiogenesis by ERK5-klf2a-nos2b Signaling. *Curr Mol Med*. 2018;18(1). doi:10.2174/1566524018666180322153432
219. Lopes SS, Lourenço R, Pacheco L, Moreno N, Kreiling J, Saúde L. Notch signalling regulates left-right asymmetry through ciliary length control. *Development*. 2010;137(21). doi:10.1242/dev.054452
220. Miura GI, Yelon D. *A Guide to Analysis of Cardiac Phenotypes in the Zebrafish Embryo*. Vol 101.; 2011. doi:10.1016/B978-0-12-387036-0.00007-4
221. Hassane S, Claij N, Lantinga-Van Leeuwen IS, et al. Pathogenic sequence for dissecting aneurysm formation in a hypomorphic polycystic kidney disease 1 mouse model. *Arterioscler Thromb Vasc Biol*. 2007;27(10). doi:10.1161/ATVBAHA.107.149252
222. Massé L, Antonacci M. Low cardiac output syndrome: Identification and management. *Crit Care Nurs Clin North Am*. 2005;17(4). doi:10.1016/j.ccell.2005.07.005
223. O'Rourke R. Mitral valve regurgitation. *Curr Probl Cardiol*. 1984;9(2). doi:10.1016/0146-2806(84)90021-5
224. Hardy MJ, Reeck JC, Fang M, Adams JS, Oxford JT. Col11a1a expression is required for zebrafish development. *J Dev Biol*. 2020;8(3). doi:10.3390/JDB8030016
225. Kodigepalli KM, Thatcher K, West T, et al. Biology and biomechanics of the heart valve extracellular matrix. *J Cardiovasc Dev Dis*. 2020;7(4). doi:10.3390/jcdd7040057
226. Drummond I. Making a zebrafish kidney: A tale of two tubes. *Trends Cell Biol*. 2003;13(7). doi:10.1016/S0962-8924(03)00124-7

227. Hoshijima K, Hirose S. Expression of endocrine genes in zebrafish larvae in response to environmental salinity. *J Endocrinol.* 2007;193(3). doi:10.1677/JOE-07-0003
228. Kumai Y, Bernier NJ, Perry SF. Angiotensin-II promotes Na⁺ uptake in larval zebrafish, *Danio rerio*, in acidic and ion-poor water. *J Endocrinol.* 2014;220(3). doi:10.1530/JOE-13-0374
229. Stainier DYR, Lee RK, Fishman MC. Cardiovascular development in the zebrafish: I. Myocardial fate map and heart tube formation. *Development.* 1993;119(1). doi:10.1242/dev.119.1.31