GLOBAL METABOLOMIC PROFILES OF AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE IN *IN VITRO* RENAL MICROCYSTS

Delaney Miller

Dr. Alyssa Hahn

BI-499 Senior Thesis

Department of Life and Environmental Sciences

Carroll College

Abstract

Autosomal-dominant polycystic kidney disease (ADPKD) is a progressive genetic disease characterized by the formation of bilateral fluid-filled cysts on the kidneys. It is one of the most common forms of chronic kidney disease (CKD), however little is understood about the mechanisms of cyst formation and propagation in renal cells. Madin-Darby Canine Kidney (MDCK) cells were cultured and stimulated with forskolin (FSK) to grow cysts in order to serve as *in vitro* models for metabolomic analysis of the pathways involved in the disease. LC-MS analysis and MetaboAnalyst testing revealed several dysregulated pathways between the experimental (n = 5; FSK+) and control (n = 5; FSK-) cohorts of MDCK cells. The most notable perturbed pathways revealed during metabolomic analysis include the carnitine shuttle, vitamin metabolism, the TCA cycle, fatty acid metabolism, and amino acid metabolism. The metabolites involved in these pathways may serve as potential targets for future therapeutic treatments of ADPKD. Results obtained from *in vitro* experiments demonstrate the value of using cell model systems for investigating the metabolic mechanisms of human disease.

Introduction

Autosomal-dominant polycystic kidney disease (ADPKD), a subclass of cystic kidney diseases known as ciliopathies, is caused by mutations in specific protein encoding genes that control the function of primary cilia. This impacts the proliferation, cell survival, polarity, and secretion of renal epithelial cells resulting in the formation of several fluid-filled cysts. Though benign, these cysts wreak havoc as they distort the morphology of the nephrons, leading to fibrosis, the destruction of nearby parenchymal cells, and eventually progressive loss of kidney function. This disease not only affects the kidneys, but several other major organs as well

including the liver, heart, pancreas, and intracerebral arteries.[1] ADPKD most commonly arises from a mutation in either the *PKD1* or *PKD2* genes which code for polycystin-1 and polycystin-2, respectively.[2] It is characterized by bilateral large kidneys that show an extensive distribution of cysts throughout. It is the most common hereditary renal disorder as well as the fourth leading cause of chronic kidney disease (CKD). The formation of renal cysts begins in utero and progresses without yield, leading to end-stage renal disease (ESRD) in approximately one-half of all afflicted patients by age 60.[2]

Though common and deadly, little is known about the mechanism of cyst formation in polycystic kidney diseases. The two genes involved in the pathogenesis of ADPKD, *PKD1* and *2*, have mutations in one allele in every cell.[2] However, not all cells form renal cysts. This leads to the assumption that a somatic mutation, occurring after conception, in either the other *PKD* allele or another gene entirely must initiate cyst formation in these already mutated cells. There are two hypotheses in the literature that work to explain this mechanism.[2] The first, a two-hit hypothesis, suggests that a complete loss of the normal allele is necessary in order for lethal cyst development. The second, a threshold hypothesis, theorizes that cysts may be able to begin to develop once the number of functional proteins fall below a certain level. This suggests that a haploinsufficiency, where a single copy of a wild-type allele is not enough to overcome the heterozygous variant allele and produce a wild-type phenotype, may be responsible for this anomaly.[2] The *PKD2* allele is highly susceptible to mutation and does show cyst development following the loss of the normal allele. Induced mutation of the second allele in vitro causes cystogenesis. It has been found that the timing of the somatic mutation event greatly influences the severity of the disease, including the basal level of proliferation and critical development window.[1] Even under normal conditions when PC1 proteins are not mutated, if their levels are

low enough (15-20%), cysts will still form. Gene mutation, protein concentration and complex interaction, and other karyotypic changes have all been shown to influence the complexity and severity of cyst development in both human and mouse models.

Previous field research has identified the roles that the polycystin genes play in the development and proliferation of renal cysts since their discovery in 1995. In typical patient populations, *PKD1* accounts for 80-85% of cases while *PKD2* accounts for the remaining 15-20%.[1] ADPKD caused by mutations in *PKD2* tend to have the best outcomes, while the disease brought on by truncated mutations of *PKD1* genes is the most fatal. Truncated proteins are shortened proteins with premature stop codons, and are therefore more harmful than the non-truncated polycystin-1 (PC1) proteins. It appears that polycystin-2 (PC2) proteins are somewhat constant, whereas PC1 proteins are metabolically regulated in tissue.[3] These two proteins react to form a PC complex, the function of which is still unknown, but which may play a role as a Ca2+ release channel in the endoplasmic reticulum of renal cells.

Recent evidence suggests that humans already have a low-full length *PKD1* mRNA caused by abnormal splicing of the *PKD1* gene that brings everyone close to the critical threshold.[4]3 With this approach, it appears that the tipping point for cystogenesis is not difficult to reach. Though the processes and mechanisms of cyst formation are still being disputed, it has been agreed upon that the three major pillars of cystogenesis are abnormal cell proliferation, incomplete differentiation, and loss of planar cell polarity.[4] In order to treat and even prevent a disease such as ADPKD, it is imperative to learn the mechanism of cyst formation so as to better understand how to combat them. This can be achieved through the science of metabolomics, where sophisticated analytical technologies are used to identify and quantify cellular metabolites that are crucial to certain pathways and processes. By mass, the kidneys only account for 0.5% of

the human body, yet they consume 10% of the body's oxygen.[5] These organs have such a high metabolic demand that when pathways are disrupted, they can start to decline quickly. Through the growth of in-vitro cysts on the Madin-Darby Canine Kidney (MDCK) cell line, we plan to identify the metabolic pathways essential to renal cyst formation so as to begin experimenting with more targeted therapies to combat such a destructive disease.

Materials/Methods

The MDCK (NBL--2) (ATCC® CCL-34™) cell line was obtained from The American Type Culture Collection and stimulated to form *in vitro* cysts as a model for ADPKD according to a previously established protocol.[2] The base medium used for cell expansion was 1:1 Dulbecco Modified Eagle Medium/Ham's F12 (DMEM/F12). MDCK cells were grown in T75 plates incubated at 37˚C and were maintained at ~70-90% confluency yielding approximately 4 x 10^6 cells per plate.

Seeding ADPKD cells in a hydrated collagen gel

MDCK cells were counted and plated in a 96-well plate at a cell suspension concentration of ~800 cells/well. After filling all perimeter wells with sterile, deionized water to prevent dehydration of the gel and medium, 60 open wells remained. These wells were filled with a solution of PureCol (3.0 mg/mL) and the cell suspension and were incubated for 45 minutes to polymerize before being treated with FSK +/- defined medium. The defined medium was a sterile solution of DMEM/F12, penicillin-streptomycin (P/S), hydrocortisone (HC), insulin/transferrin/selenium (ITS), and triiodo-l-thyronine (T3).

The experimental microcyst wells (n=30) were treated with the agonist forskolin (FSK) to initiate cyst formation as well as defined medium to supply cell nutrients. FSK is a cell-

permeable diterpene that directly activates adenylyl cyclase, the enzyme that produces cyclic adenosine monophosphate (cAMP) which raises cAMP levels in the cell.[2] The control wells (n=30) were treated with FSK- defined medium so as to control for the effects of P/S, HC, ITS, and T3. After 24 hours, the media was removed from all wells and replaced with either a lower concentration FSK+ defined media solution or pure defined media (FSK-) so as to reduce the persistent effects of the agonist during the experiment. Cysts were then given an additional four days to grow before image capturing and metabolomic analysis.

Photographing Cysts

Cyst growth development was monitored via optical microscope and images were captured and measured using an AmScope MU USB 2.0 camera and its accompanying Windows software disc. Subsequently, all 60 experimental and control wells were analyzed for contamination and cystogenesis. A total of ten wells (n=5 experimental, FSK+; n=5 controls, FSK-) were selected for further metabolomic analysis.

Metabolite Extraction and Mass Spectrometry

Metabolites were extracted using our previously established protocol with some modifications.[6] Well contents were weighed, extracted, submerged in 3:1 methanol:water, and homogenized in a tissue grinder for 4 cycles of 25 seconds each at 3000. Samples were centrifuged to remove cell debris, proteins were precipitated with 4 volumes of acetone, and supernatant was removed and frozen with 8:2 methanol:milliQ H_2O for metabolite extraction. Supernatant was subsequently dried down in a vacuum concentrator for 2 hours. Metabolites were resuspended using mass spectrometry grade 50:50 water:acetonitrile solution and run through LC-MS. Metabolites extracted from FSK+ and FSK- wells were analyzed using our previously established LC-MS analysis protocol.[6] Briefly, metabolites were analyzed using an

Agilent 1290 UPLC system connected to an Agilent 6538 Q-TOF mass spectrometer in normal phase using a HILIC column.

Statistical Analyses

Data generated by the mass spectrometer were processed as previously described.[6] Statistical analyses were performed in the metabolomics data analysis platform, MetaboAnalyst.[7,8] Prior to statistical analyses, data were log transformed and standardized. Differences between FSK+ and FSK- cohorts were assessed using hierarchical cluster analysis (HCA), clustergrams, volcano plot analysis, Student's T-test, principal component analysis (PCA), and partial least squares-discriminant analysis (PLS-DA). Metabolite identity matches and enriched pathways were identified using MetaboAnalyst MS Peaks to Pathways.

Results

There are noticeable differences in the metabolisms between FSK+ and FSK- wells based on statistical analyses performed. MDCK cells were stimulated to grow in vitro microcysts via addition of FSK to defined medium treatment. The presence of cysts was confirmed through microscopy and the measurement of cyst sizes across FSK+ and FSK- wells. Unsupervised statistical analysis HCA, showed the majority of samples clustered within their respective cohorts, showcasing distinct metabolomic profiles in each cohort. Only a single sample in each cohort was incorrectly classified by HCA (Figure 1A-B).

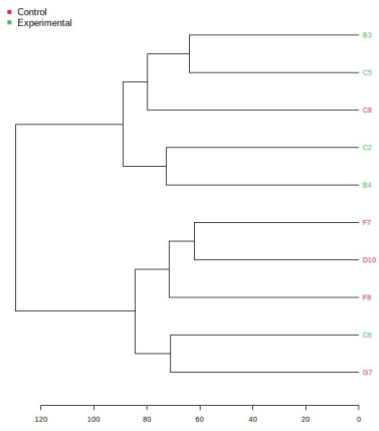

Figure 1A. Dendrogram illustrating separation between FSK stimulated and unstimulated wells. Unsupervised clustering revealed that four out of five samples clustered with their respective cohorts.

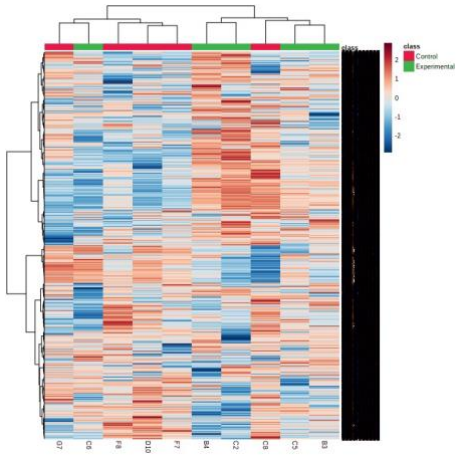

Figure 1B. Clustergram visualizes overall global metabolomic profiles. Heatmap shows clustering of samples within their respective cohorts (top) and reveals groups of coregulated metabolites (side)

A second unsupervised statistical analysis, PCA, further confirmed the shift in metabolism between cells stimulated with FSK+ and those that were not (Figure 2). These results suggest that cells forming cysts have noticeable metabolic perturbations.

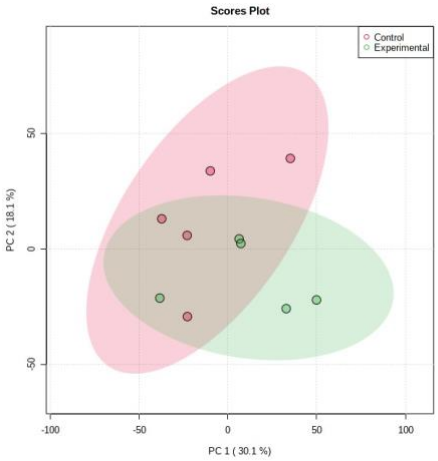

Figure 2. Some separation in metabolomic profiles between FSK stimulated and unstimulated wells in unsupervised PCA. PCA shows natural separation between cohorts. PCA reveals some overlap in metabolomic profiles, with principal components 1 and 2 accounting for 48.2% of the variability of the dataset.

Supervised statistical analysis PLS-DA, again solidified the distinct metabolomic profiles between FSK+ and FSK- samples (Figure 3). All samples were correctly classified within their

respective cohorts (Figure 3). PLS-DA assigns scores for metabolites that contribute the most to the separation between cohorts, termed Variable Importance in Projection (VIP) scores. The metabolites with the highest VIP scores were mapped to metabolic pathways using MetaboAnalyst MS Peaks to Pathways.[7,8]

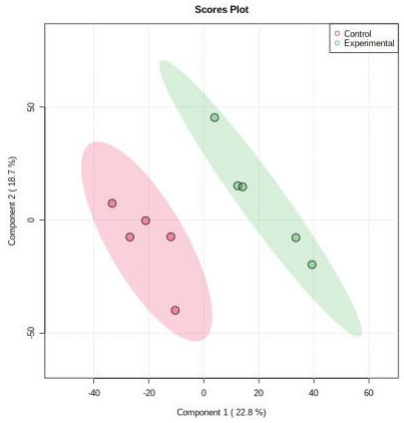

Figure 3. Distinct metabolomic profiles between FSK stimulated and unstimulated wells in supervised PLS-DA. PLS-DA seeks out the differences between cohorts. PLS-DA shows clear separation between FSK stimulated and unstimulated wells. Components 1 and 2 account for 41.5% of the variability of the dataset.

These metabolites subsequently mapped to the a number of pathways listed in Table 1, most notably the carnitine shuttle, vitamin metabolism, the TCA cycle, fatty acid metabolism, and amino acid metabolism (Table 1).

Table 1. Enrichment analysis. Metabolite features with the highest variable importance in projection (VIP) scores in PLS-DA were mapped to metabolic pathways to reveal those that contributed to the driving differences between cohorts

Pathway Name	Total	Hits (all)	Hits (sig.)
Carnitine shuttle	72	31	2
Vitamin D3 (cholecalciferol) metabolism	16	2	1
Bile acid biosynthesis	82	2	1
Vitamin B9 (folate) metabolism	33	5	1
Valine, leucine, and isoleucine degradation	65	8	1
Butanoate metabolism	34	4	1
Ascorbate (Vitamin C) and alderate metabolism	29	3	1
Fatty acid oxidation, peroxisome	28	14	1
TCA cycle	31	3	1
Arginine and proline metabolism	45	10	1
Glutamate metabolism	15	1	1
Porphyrin metabolism	43	14	1
Pyrimidine metabolism	70	4	1
Starch and sucrose metabolism	33	7	1

Discussion

Taken together, these results suggest that the MDCK cells stimulated with FSK have a distinct shift in metabolism compared to MDCK cells that were not stimulated with FSK. The metabolic pathways identified by PLS-DA VIP score enrichment may be indicative of metabolic shifts in PKD. Specifically, the metabolic pathways of the carnitine shuttle, vitamin B9 and C, fatty acids, the TCA cycle, and amino acids may have implications in PKD cystogenesis.

Carnitine shuttle

Fatty acids are used for energy when glucose is unavailable.[9] Via the carnitine shuttle, long fatty-acid chains that are impermeable to the mitochondrial membranes are transported into the matrix for beta-oxidation and energy production.[9] The secondary function of the shuttle is to regulate the intramitochondrial ratio of acyl coenzyme A to free coenzyme A.[9] Renal cells rely heavily on the beta-oxidation of fatty acids because the kidneys are two of the most high-energy demanding organs in the body (second only to the heart in mitochondrial abundance).[10] Genome-wide transcriptome analysis in previous studies has identified FAO as the top dysregulated pathway in kidney diseases (decreased expression of key FAO enzymes and regulators).[10] It has also been found that stimulation of CPT1 (carnitine palmitoyltransferase type 1) has a protective effect against renal fibrosis.[10] Carnitine palmitoyltransferase deficiency type 2 (CPT2) has been associated with cystic kidneys that are especially severe in infants.[9] Additionally, mitochondrial dysfunction is commonly observed across all nephropathies.[10]

Vitamin B9 (folate) metabolism

Previous studies have noted the consequences of abnormal vitamin B9 (folate) levels on kidney function, especially for those with a comorbid nephropathy and diabetes. Specifically, folate has been implicated as a contributing factor to renal anemia and hyporesponsiveness to

certain therapeutic treatments.[11] Folate plays a critical role in the anabolism of nucleic acid precursors and several amino acids. Its coenzymes are involved in two key pathways of DNA metabolism: the synthesis of DNA from its precursors and the conversion of homocysteine to methionine. Methionine is required to synthesize the methyl group donor S-adenosylmethionine (SAM) which is the key player in almost all biological methylation reactions. As a result of this cascade that starts with folate, DNA, RNA, proteins, and phospholipids become methylated. This precise methylation of DNA especially controls gene expression as well as certain cell differentiation processes.[11] Dysregulation of metabolites involved in folate metabolism may impact the initiation and/or growth of cystic renal cells as indicated by one significant hit in Table 1. The result of unmethylated DNA in renal cells may be that unchecked gene expression ultimately aids cyst propagation.

Valine, leucine, and isoleucine degradation

The branched chain amino acids (BCAAs) valine, leucine, and isoleucine are essential amino acids that serve as substrates for protein synthesis and energy production. Specifically, they exert a stimulatory effect on protein anabolism and an inhibitory effect on proteolysis. Increased activity of the enzyme branched-chain α-keto acid dehydrogenase (BCKD) has been found to contribute to the enhanced oxidation of BCAAs seen in chronic renal failure (CRF).[12] The metabolic precursors involved in the BCAA degradation process likely contribute to the growth and/or propagation of renal cysts in cases of ADPKD. There is promising potential for CRF therapy to include BCAA supplementation coupled with a low protein diet, but more studies are needed to better understand their effectiveness.[12]

Ascorbate (Vitamin C) and Aldarate metabolism

All eukaryotes, the major exception being primates, are able to synthesize ascorbic acid naturally. Primates and some other animal groups lost the functional gulono-lactone oxidase (GULOP) gene and have since learned to supplement with vitamin-C rich diets. Its conjugate base, ascorbate, acts as an electron donor and free radical scavenger which reduces higher oxidation states of iron to Fe^{2+}. These reactions create relatively stable radicals of monodehydroascorbate, deactivating the pathway that would turn off the essential 2-oxoglutarate-dependent-dioxygenases (2-ODDs).[13] These enzymes have diverse functions including the methylation of histones and DNA, the dysregulation of which is greatly implicated in the formation of renal cysts. Unfortunately, because ascorbate is such a strong antioxidant, it can end up overperforming by reacting in the presence of transition metals like Fe^{2+} and producing excess oxidants which cause extra stress on uremic patients.[14] The dysregulation observed in ascorbate metabolism of ADPKD cells parallels the low plasma ascorbate levels seen in CKD patients, which is part of the reason vitamin C supplementation is so essential in treating kidney disease. Ultimately, metabolites involved in the ascorbate pathways showed significant perturbation between cohorts indicating that vitamin C is a key player in cyst formation.

Fatty acid oxidation, peroxisome

Fatty acid oxidation has long been a player in the field of CKD research as it is largely associated with renal fibrosis, the scarring characteristic of end-stage kidney disease. Specifically, individuals with tubulointerstitial fibrosis show decreased expression of necessary enzymes and regulators of fatty acid oxidation (FAO) resulting in higher intracellular lipid concentrations as compared to healthy controls.[15] This buildup results in ATP depletion, cell death, and dedifferentiation, all symptoms of renal fibrosis. In ADPKD, loss of the *PKD* gene

causes a cascade of metabolic changes that affect glycolysis, mitochondrial metabolism, and fatty acid synthesis which antagonizes cell growth and survival.[16] Via transcriptomics, urine metabolomics, and lipidomics, reduced fatty acid oxidation has been identified as a key feature in ADPKD.[9] Two proteins, hepatocyte nuclear factor 4α (Hnf4α) and peroxisome proliferator-activated receptor-α (PPARα), are responsible for several metabolic programming pathways and have been indicated as central to the reduced FAO seen in PKD. Treatment with fenofibrate, a PPARα agonist, has been shown to enhance FAO and reduce cystogenesis while loss of the Hnf4α protein increases the severity of cystic diseases.[17] Other metabolites involved in the fatty acid oxidation pathways may prove to be future targets for therapeutic treatments in ADPKD cases.

TCA cycle

The tricarboxylic acid (TCA) cycle, also known as the Krebs or citric acid cycle, is a process of aerobic respiration that releases stored energy from carbohydrate, fat, and protein derivatives. The phenomenon of mitochondrial metabolic impairment noted across all CKDs confirms that metabolites in the pathways taking place inside or in conjunction with these organelles are severely dysregulated.[17] In stage 3-4 CKD patients, urine metabolomic analysis reveals that the TCA cycle is the most significantly perturbed pathway due to the excretion of citrate, cis-aconitate, isocitrate, 2-oxoglutarate, and succinate being reduced by 40-68%.[18] Additionally, in kidney biopsies of the same stage patients, the expression of genes regulating many of these compounds was found to be significantly reduced.[18] This, as well as the dysregulation of the TCA cycle observed in our *in vitro* cysts, supports the multitude of emerging evidence suggesting that CKD is overall a disorder of mitochondrial dysfunction. It

should also be noted that mutant *PKD1* cells preferentially use glutamine to fuel the TCA cycle which ultimately disrupts cell growth and survival.[16]

Arginine and proline metabolism

The metabolism of arginine and proline is bidirectional and links arginine, glutamate, and proline into one of the most prolific biosynthetic pathways in the human body. The kidneys are the main site of L-arginine production in the body and are namely responsible for arginine synthesis, creatine synthesis, and arginine reabsorption.[19] L-arginine is a semi-essential amino acid as well as a substrate for nitric oxide synthesis. These metabolites are highly indicated in pathogenesis of CKDs, the dietary modification of which is often used as in treatment.[19] In general, the renal metabolism of amino acids is highly dysregulated in kidney diseases. Previous studies of CKD patients show that renal uptake of the amino acids glutamate and proline and the release of serine and threonine is reduced by 80-90% and the uptake of phenylalanine and release of ornithine stops completely.[20] Additionally, the uptake of citrulline and release of alanine, arginine, lysine, and tyrosine is reduced by over 60%. The exchange of the remaining essential amino acids via red blood cells disappears resulting in an significant overall reduction in ammonia production.[20] The pathways involving amino acid metabolism, specifically arginine and proline, prove to be significant contributors of renal dysfunction and uremia.

Glutamate metabolism

The metabolism of glutamate, a non-essential amino acid, is vital in the production of proteins and nucleic acids. Glutamate is a precursor molecule for various metabolites as well as the essential amino acids L-proline and L-arginine.[21] In healthy kidneys, glutamate filtration and metabolism is extremely efficient, resulting in very little glutamate being excreted in urine. In individuals with advanced stages of CKD, higher levels of glutamate are observed in their

plasma, indicating that the elimination of excess glutamate may be inhibited.[22] Excessive glutamate in the kidney is toxic and if the catabolic metabolites involved in this pathway are perturbed, cystogenesis can occur.

This is a preliminary analysis of the global metabolomics dataset generated by this study. The results suggest that stimulation by FSK induces a shift in cellular metabolism that may be driving cyst formation. The most notable metabolic pathways that were perturbed between FSK-stimulated and unstimulated wells were the carnitine shuttle, vitamin metabolism, fatty acid metabolism, the TCA cycle, and amino acid metabolism. These pathways and their derivatives may prove to be drivers of cyst formation in ADPKD. The results of this study suggest that renal cyst formation is associated with metabolic reprogramming and differential gene expression. Future studies should increase sample size between experimental and control groups. It would also be necessary to create a control cohort that examines only the effects of FSK on 2D MDCK cells to rule out whether or not the identified pathways are simply linked to FSK stimulation. This would validate that the metabolic pathways implicated in the study can serve as targets for therapeutic treatment methods. A greater understanding of metabolism in disease pathogenesis may lead to the identification of potential drug targets to block or reverse not only the pathways towards cyst formation in ADPKD but the aggregates of a variety of renal disorders and nephropathies.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Ong, Albert C.M, & Harris, Peter C. (2015). A polycystin-centric view of cyst formation and disease: The polycystins revisited. *Kidney International, 88*(4), 699-710.

2. Sharma, M. (2019). In vitro cyst formation of ADPKD cells. *Methods in Cell Biology.*, 93-111.
3. Nigro EA, Castelli M, Boletta A. Role of the Polycystins in Cell Migration, Polarity, and Tissue Morphogenesis. Cells. 2015 Oct 30;4(4):687-705.
4. Lea, Wendy A, et al. (2020). Analysis of the polycystin complex (PCC) in human urinary exosome-like vesicles (ELVs). *Scientific Reports, 10*(1), 1500.
5. Nowak, Kristen L, & Hopp, Katharina. (2020). Metabolic Reprogramming in Autosomal Dominant Polycystic Kidney Disease: Evidence and Therapeutic Potential. *Clinical Journal of the American Society of Nephrology, 15*(4), 577-584.
6. Carlson, Alyssa K, Rawle, Rachel A, Adams, Erik, Greenwood, Mark C, Bothner, Brian, & June, Ronald K. (2018). Application of global metabolomic profiling of synovial fluid for osteoarthritis biomarkers. *Biochemical and Biophysical Research Communications, 499*(2), 182-188.
7. Jianguo Xia, Nick Psychogios, Nelson Young, David S. Wishart, MetaboAnalyst: a web server for metabolomic data analysis and interpretation, *Nucleic Acids Research*, Volume 37, Issue suppl_2, 1 July 2009, Pages W652–W660.
8. Jianguo Xia, David S. Wishart, MetPA: a web-based metabolomics tool for pathway analysis and visualization, *Bioinformatics*, Volume 26, Issue 18, 15 September 2010, Pages 2342–2344.
9. El-Gharbawy, A., & Vockley, J. (2018). Inborn Errors of Metabolism with Myopathy: Defects of Fatty Acid Oxidation and the Carnitine Shuttle System. *Pediatric clinics of North America, 65*(2), 317–335.

10. Console, L., Scalise, M., Giangregorio, N., Tonazzi, A., Barile, M., & Indiveri, C. (2020). The Link Between the Mitochondrial Fatty Acid Oxidation Derangement and Kidney Injury. *Frontiers in physiology*, *11*, 794.
11. Teschner, M., Kosch, M., & Schaefer, R. M. (2002). Folate metabolism in renal failure. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*, *17 Suppl 5*, 24–27.
12. Holeček, M. Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. *Nutr Metab (Lond)* 15, 33 (2018).
13. Smirnoff N. (2018). Ascorbic acid metabolism and functions: A comparison of plants and mammals. *Free radical biology & medicine*, *122*, 116–129.
14. Deicher, R., & Hörl, W. H. (2003). Vitamin C in chronic kidney disease and hemodialysis patients. *Kidney & blood pressure research*, *26*(2), 100–106.
15. Kang, H., Ahn, S., Choi, P. *et al.* Defective fatty acid oxidation in renal tubular epithelial cells has a key role in kidney fibrosis development. *Nat Med* 21, 37–46 (2015).
16. Podrini, C., Rowe, I., Pagliarini, R. *et al.* Dissection of metabolic reprogramming in polycystic kidney disease reveals coordinated rewiring of bioenergetic pathways.*Commun Biol* 1, 194 (2018).
17. Jia Z. (2017). Impaired Citric Acid Cycle in Nondiabetic Chronic Kidney Disease. *EBioMedicine*, *26*, 6–7.
18. Hallan, Stein, Afkarian, Maryam, Zelnick, Leila R, Kestenbaum, Bryan, Sharma, Shoba, Saito, Rintaro, . . . De Boer, Ian H. (2017). Metabolomics and Gene Expression Analysis Reveal Down-regulation of the Citric Acid (TCA) Cycle in Non-diabetic CKD Patients. *EBioMedicine, 26*(C), 68-77.

19. Cherla, G., & Jaimes, E. A. (2004). Role of L-arginine in the pathogenesis and treatment of renal disease. *The Journal of nutrition*, *134*(10 Suppl), 2801S–2819S.
20. Tizianello, A., De Ferrari, G., Garibotto, G., Gurreri, G., & Robaudo, C. (1980). Renal metabolism of amino acids and ammonia in subjects with normal renal function and in patients with chronic renal insufficiency. *The Journal of clinical investigation*, *65*(5), 1162–1173.
21. Yelamanchi, S. D., Jayaram, S., Thomas, J. K., Gundimeda, S., Khan, A. A., Singhal, A., Keshava Prasad, T. S., Pandey, A., Somani, B. L., & Gowda, H. (2016). A pathway map of glutamate metabolism. *Journal of cell communication and signaling*, *10*(1), 69–75.
22. Du, Jie, Li, Xiao-Hui, & Li, Yuan-Jian. (2016). Glutamate in peripheral organs: Biology and pharmacology. *European Journal of Pharmacology, 784,* 42-48.