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IDENTIFICATION OF NEW BIOMARKERS FOR DISEASE PROGRESSION IN AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE

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Dedication

This thesis is dedicated to the memory of my parents

(Abokhzam Mohammed and Omalsad Salem)

ACKNOWLEDGEMENT

All praise is to Almighty Allah for making this work and every work possible.

It was a great privilege to work under the supervision of Professor Albert Ong and my sincerest appreciation is to him for his mentorship and tremendous support throughout the course. I am most grateful to my teacher Dr Andrew Streets, for training me to conduct laboratory experiments, valuable discussion, advice, support and exceptional patience. I wish to express my sincere thanks to Dr Roz Simms for her wonderful support and invaluable help in recruiting patients from clinic. I acknowledge Dr Paul Heath and his great team (Mr Mathew Wyles and Dr Rachel Waller) for helping me with next generation sequencing and for their detailed explanation of each step in the experiments. I wish to thank all the members of the Kidney Genetics Group who contributed to my training especially Mrs Fiona Wright, all colleagues and administrative staff in the Academic Unit of Nephrology. Special thanks to Dr Jiehan Chong for his help with blood sampling from healthy volunteers. I express my deep gratitude to all study participants for their valuable contribution to this project. I acknowledge Dr Jean Russell, at the Corporate Information and Computing service, for her kind help with the statistical analysis and thank Dr Bart Wagner for his skilled assistance with electron microscopy.

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Hypothesis

The discovery of new urine biomarkers will allow the development of tests to identify ADPKD patients at risk of rapid disease progression for therapy and pinpoint the key pathways underlying disease progression.

Aims and Objectives

The major aims of this project were to:

1. Identify exosome-associated proteins that correlated with the rate of disease progression in ADPKD.

2. Identify exosome-associated urinary microRNAs altered in ADPKD patients compared to healthy controls.

3. Profile urine angiogenic factors altered in ADPKD and their potential role in disease progression.

Abstract

Introduction: ADPKD is the most common renal genetic disease and the fourth most common cause of end-stage renal disease (ESRD) world-wide. Although *PKD1* and *PKD2* patients have different phenotypes, there is also significant intra-familial variability in disease progression suggesting that other genetic or environmental factors have major influences on disease progression. With the availability of new therapies, there is an urgent need to identify novel biomarkers which can identify ADPKD patients at risk of rapid disease progression at an early stage.

Methods: Spot urine specimens were collected from consecutive patients with ADPKD (n = 130) attending a PKD clinic at the Sheffield Kidney Institute and healthy controls (n = 33). Urinary exosomes were isolated from urine samples by ultracentrifugation and confirmed by electron microscopy and western blotting. The expression of exosomes associated proteins, namely polycystin-1, polycystin-2 and ErbB4, cell free microRNAs, exosomes associated microRNAs and angiogenesis related proteins were examined in spot urine samples from healthy volunteers, ADPKD patients with early (eGFR > 60 ml/min) or late (eGFR < 60 ml/min) disease using western blotting, quantitative PCR, next generation sequencing, angiogenesis proteomics array and ELISA.

Results: Electron microscopy confirmed the presence of multiple (< 100 nm) vesicles in the pelleted fraction and western blotting confirmed that the exosome specific protein TSG-101 was only detectable in the pellet. A significant decrease in exosome associated PC1 and PC2, normalised for TSG-101, was clearly detected in ADPKD patients with both early and late disease compared to healthy volunteers. I also detected an ErbB4 positive band at 80 kDa, corresponding to the known C-terminal intracellular domain (ICD) generated by γ-secretase mediated cleavage. In contrast to PC1 and PC2, I found that expression of the 80 kDa band was significantly increased in ADPKD patients with late disease compared to healthy controls as defined by baseline eGFR. These changes in ErbB4 confirm our recent findings in human cystic cells and murine Pkd1 tissue.

Cell free urine contains very low levels of microRNAs compared to exosomes. A specific set of microRNAs was found downregulated in urine samples from patients with ADPKD when compared to the controls, including miR-30 family, miR-192-5p, miR-193b-3p and miR-194-5p. The differentially expressed microRNAs were found negatively correlated with eGFR and positively correlated with mean kidney length, and

were significantly better predictors of the rate of disease progression in ADPKD compared to mean kidney length and together had a combinatory effect.

In addition, a specific signature for angiogenesis related proteins was found in urine samples from patients with ADPKD compared to healthy controls. Relative expression of MCP-1 in urine is significantly correlated with eGFR and mean kidney length, and it is a significantly better predictor of ADPKD progression compared to mean kidney length.

Conclusions: This study has shown that exosomes purified from ADPKD patients are a promising source of urinary biomarkers. Progressive ADPKD is associated with alteration in urine profiling of exosomes associated proteins, microRNAs as well as angiogenesis related factors. ErbB4, miR-30 family, miR-192-5p and miR-193b-3p were the best markers for disease progression (eGFR slope) outperforming mean kidney length. The discovery of distinct biomarker profiles will allow detection of those at high risk of disease progression so as to identify those who might benefit from earlier and more intensive intervention.

Publication

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Oral presentations

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Abbreviations

ACEI	Angiotensin converting enzyme Inhibitor
ACR	Urine albumin to creatinine ratio
ADPKD	Autosomal Dominant Polycystic Kidney Disease
ADPLD	Autosomal Dominant Polycystic Liver Disease
AKI	Acute kidney injury
ANCA	Anti-neutrophil cytoplasmic antibodies
ANOVA	Analysis of variance
AQP-2	Aqporin-2
ARB	Angiotensin receptor blocker
ARPKD	Autosomal Receive Polycystic Kidney Disease
ATP	Adenosine triphosphate
AUC	Area under an ROC Curve
BMI	Body Mass Index
BSA	Bovine Serum Albumin
cAMP	3”, 5”- cyclic adenosine monophosphate
cDNA	Complementary DNA
CE-MS	Capillary electrophoresis coupled online to mass spectrometry
CFTR	Cystic fibrosis transmembrane conductance regulator
CKD	Chronic Kidney Disease
CKD-EPI	Chronic kidney disease epidemiology collaboration
CRISP	Consortium for Radiologic Imaging Studies of Polycystic Kidney Disease
Ct	Cycle threshold
CT-scan	Computed tomography scanning
DDT	Dichlorodiphenyltrichloroethane

DNA	Deoxyribonucleic acid
dNTP	deoxynucleotriphosphate
DM	Diabetic mellitus
ECM	Extracellular matrix
EGF	Epidermal growth factor
eGFR	estimated Glomerular Filtration Rate
EGFR	Epidermal growth factor Receptor
ELISA	Enzyme Linked Immunosorbent assay
ER	Endoplasmic reticulum
ESRD	End Stage Renal Disease
HALT-PKD	Halt progression of polycystic kidney disease
HB-EGF	Heparin-binding EGF-like growth factor
HDL	High-density lipoprotein
HTN	Hypertension
IgA nephropathy	Immunoglobin A nephropathy
IL	Interleukin
JAK	Janus kina
kb	Kilobase
kDa	Kilodalton
M	Molar
MAB	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MDCK	Madin-Darby Canine Kidney Cells
mg	milligram

µg	microgram
MiR	MicroRNAs
MKL	Mean kidney length
ml	millilitre
µl	microliter
MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
mTOR	mammalian Target of Rapamycin
MVB	Multivesicular bodies
ng	nanogram
NGAL	Neutrophil gelatinase-associated lipocalin
NGS	Next Generation Sequencing
NF	Nuclear factor
NFAT	Nuclear factor of activated T-cells
NSAID	Non-steroidal anti-inflammatory drugs
nt	nucleotide
PAGE	Polyacrylamide gel electrophoresis
PC-1	Polycystin-1
PC-2	Polycystin-2
PCR	Protein Creatinine Ratio
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived endothelial cell growth factor
PI3K	Phosphatidylinositol 3 kinase
PKA	Protein kinase A
PKD	Polycystic kidney disease

PLD	Polycystic liver disease
PTH	Parathyroid hormone
qPCR	quantitative Polymerase Chain Reaction
RBF	Renal blood flow
RNA	Ribonucleic acid
rRNA	ribosomal RNA
ROC curve	Receiver operating characteristic curve
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecylsulphate
SEM	Standard Error of the Mean
SLE	Systemic Lupus Erythematosus
SSTR	Somatostatin receptor
STAT	Signal Transducers and Activators of Transcription
TBS	Tris Buffer Saline
TGF	Transforming Growth Factor
THP	Tamm Horsfall Protein
TKV	Total Kidney Volume
TLV	Total Liver Volume
Tm	melting Temperature
tRNA	transfer RNA
TRV	Total Renal Volume
TSG-101	Tumour Susceptibility Gene-101
US	Ultrasonography
VEGF	Vascular Endothelial Growth Factor

Chapter 1 Introduction

1.1 ADPKD

ADPKD is a genetic disease characterized by bilateral cyst formation in the kidneys and other organ cysts such as the liver, pancreas and arachnoid membrane (**Figure 1.1**) (Wu and Somlo 2000; Torres, Harris et al. 2007). It is the fourth leading cause of end stage renal disease (ESRD), after diabetes, hypertension and glomerulonephritis (Helal, Reed et al. 2012). It accounts for approximately 8-10% of patients on renal replacement therapy (Ong and Devuyst 2011; Helal, Reed et al. 2012).

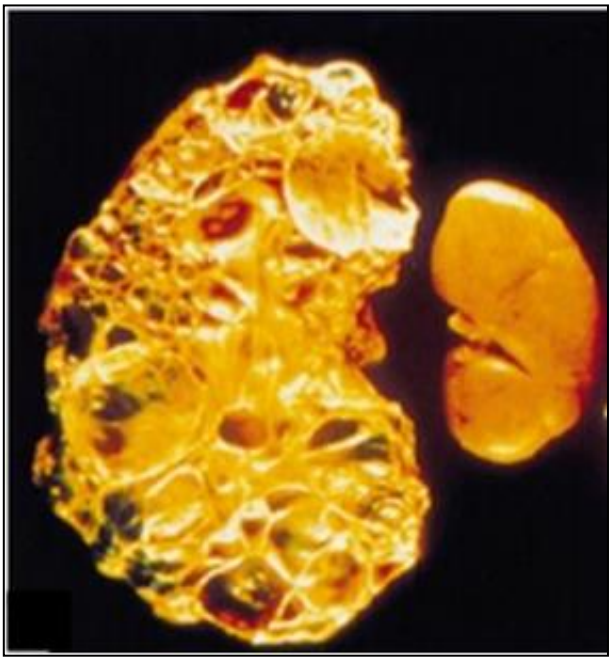

Figure 1.1. ADPKD kidney compared to a normal kidney

The figure compared kidney from patients with ADPKD and a normal kidney. The figure was modified from Wu et al. (Wu and Somlo 2000) with permission.

1.2 Epidemiology

ADPKD is a disease affecting all races with a prevalence of 1:400 to 1:1000 (Ong and Harris 2005; Torres, Harris et al. 2007). It progresses into ESRD between the fourth and six decades of life (Helal, Reed et al. 2012). The disease is more aggressive in men compared to women with a ratio of 1.2 to 1.3 at ESRD (Torres, Harris et al. 2007). Roughly 50% of ADPKD patients need renal replacement therapy around the age of sixty (Wolyniec, Jankowska et al. 2008). A retrospective study by Lentine et al. (2010), found that the healthcare cost of US patients with ADPKD is significantly influenced by the changes in baseline renal function. The mean medical annualized charges

increased by approximately 5 fold in patients with advanced renal impairment (eGFR < 15 ml/min/1.73^{2}m) in comparison with patients with stable renal functions (eGFR > 90 ml/min/1.73^{2}m) (Lentine, Xiao et al. 2010).

1.3 ADPKD genes

ADPKD results from mutations in one of two genes: *PKD1,* on chromosome [16 p13.3] and *PKD2*, on chromosome [4q21] (Hateboer, v Dijk et al. 1999). Approximately 85% of ADPKD patients have *PKD1* mutations, whereas 15% have mutations in *PKD2* (Pei and Watnick 2010). *PKD1* has 46 exons spread over 52 kb of DNA (Thomas, McConnell et al. 1999). The large size of the gene makes it vulnerable to variety of mutations which truncate the protein via nonsense, insertions or deletions, splicing changes and missense or in-frame (Rossetti, Strmecki et al. 2001). *PKD2* comprises 15 exons extending over 68 kb of genomic DNA (Harris and Torres 2009). Unlike *PKD1*, the majority of *PKD2* mutations result in truncating mutations or null alleles (Veldhuisen, Saris et al. 1997; Rossetti, Strmecki et al. 2001). However, both diseases are indistinguishable clinically as both genes are expressed in the same tissues (Pei and Watnick 2010). It has been reported that the mean age of developing renal failure of *PKD1* mutation's individuals is approximately 54.3 years, whereas the mean age of ESRD onset in *PKD2* patients is around 74 years (Hateboer, v Dijk et al. 1999). Approximately 8-10% of families with ADPKD have an unknown mutation (Heyer, Sundsbak et al. 2016; Mao, Chong et al. 2016). Recently, it was reported that mutations in GANAB can give rise to an ADPKD or ADPLD phenotype (Porath, Gainullin et al. 2016). The disease mechanism is most likely driven by defective PC1 and PC2 maturation in the endoplasmic reticulum (Porath, Gainullin et al. 2016).

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1.4 ADPKD proteins

The ADPKD proteins have been named polycystin 1 and 2 (Burn, Connors et al. 1995; Mochizuki, Wu et al. 1996). Polycystin 1 is a large integral membrane glycoprotein of approximately 460 kDa and consists of 4302 amino acids (Harris 1999). It has the typology of an adhesion molecule or orphan receptor with a large extracellular N region, short intracellular C region and 11 transmembrane regions (**Figure 1.2**) (Hughes, Ward et al. 1995; Torres, Harris et al. 2007). The long extracellular N region (approximately 3000 amino acids) contains several domains, including a receptor for egg jelly (REJ domain). The REJ domain is predicted to mediate protein–carbohydrate or protein–protein interaction (Veldhuisen, Spruit et al. 1999). The cytoplasmic C-terminus has around 200 amino acids and contains an alpha-helical coiled-coil structure which interacts with C-terminus of PC2. In addition, it contains a number of phosphorylation sites which may mediate functional interactions between various intracellular signalling molecules and PC1. Several groups have reported the localisation of PC1 at the basolateral plasma membrane, adhesion junctions, desmosomes, primary cilia as well as in the nucleus, endoplasmic reticulum and exosomes (Torres, Harris et al. 2007; Ong and Harris 2015). PC1 has been shown to control the stability of actin-associated adherent junctions and may play a role in the regulation of cell adhesion (Huan and van Adelsberg 1999; Roitbak, Ward et al. 2004). Based on its predicted structure; it was proposed that PC1 mediates cell-matrix or cell-cell interactions (Hughes, Ward et al. 1995; Wilson, Geng et al. 1999). In addition, there is evidence that PC1 may function as a mechanosensory receptor in cilia (Yoder, Hou et al. 2002; Nauli, Alenghat et al. 2003).

Polycystin 2 is a 110 kDa protein of 968 amino acids and contains short cytoplasmic N-terminal and C-terminal regions with 6 intervening transmembrane domains (Harris 1999) (**Figure 1.2**). PC2 is localized predominantly in the endoplasmic reticulum, but a small percentage has also found in the primary cilium and plasma membrane (Torres and Harris 2009). It has sequence homology to transient receptor potential (TRP) channels which are thought to function as non-selective calcium channels (Newby, Streets et al. 2002; Chang and Ong 2008). PC1 and PC2 interact through coil-coiled domains located at their cytoplasmic C-terminal to form a functional complex (Qian, Germino et al. 1997; Hateboer, Veldhuisen et al. 2000). A major function of the polycystin complex is to regulate intracellular calcium balance (Pei and Watnick 2010).

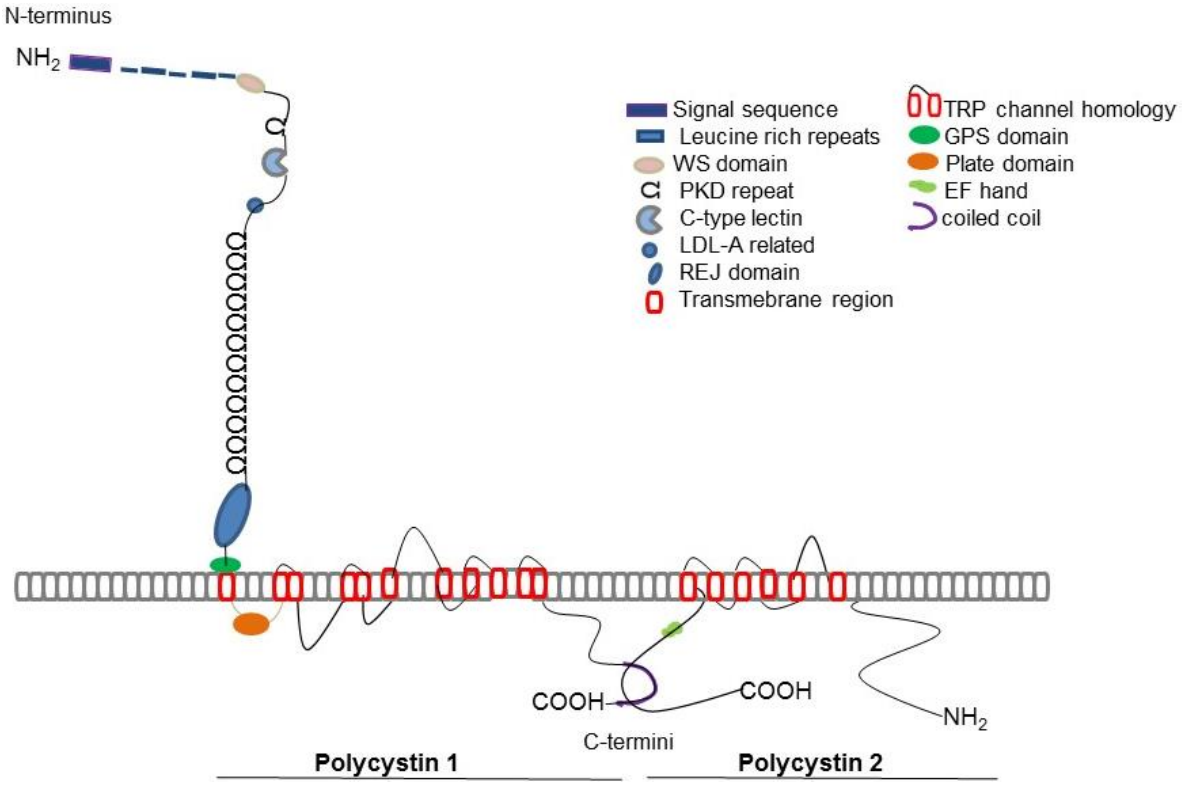

Figure 1.2. Proposed structure of polycystins

PC1 has the configuration of adhesion molecule or a receptor and has a large extracellular N region, short intracellular C region and 11 transmembrane regions. PC2 contains short cytoplasmic N-terminal associated with a ciliary targeting motif, short C-terminal region and 6 transmembrane domains. GPS is a potential proteolytic cleavage site domain (Mengerink, Moy et al. 2002).

1.5 Cystogenesis

There is still debate as to the precise mechanism of cystogenesis, specifically whether a partial or complete loss of normal allele function is required to trigger cyst growth (Ong and Harris 2015). A complete loss of PKD function is the consequence of a two hit mechanism, and this mechanism requires a germline mutation followed by a somatic mutation of either of the PKD genes in cystic epithelial cells (Somlo and Markowitz 2000; Ong and Harris 2015). The second possibility is the threshold mechanism; reduction of PKD protein levels below a specific dosage level maybe sufficient to trigger cystogenesis in cells with a germline mutation (Lantinga-van Leeuwen, Dauwerse et al. 2004; Ong and Harris 2015).

Whatever the precise mechanism, defects in polycystin function result in cyst formation from normal nephrons (Grantham, Geiser et al. 1987; Torres and Harris 2009) (**Figure 1.3**). Early features of this process are associated with cellular alterations in proliferation, differentiation, apoptosis, extracellular matrix remodelling, apical basal polarity, fluid secretion and tubular cilia abnormality (Chang and Ong 2008; Torres and Harris 2009). In addition, various signalling pathways have been implicated in the pathogenesis of cystogenesis (**Figure 1.4**). The growing cyst leads to compression of the surrounding parenchyma tissue, vascular remodelling and also releases cytokine and chemokine that stimulate fibrosis and disease progression (Bello-Reuss, Holubec et al. 2001; Grantham and Torres 2016). How previously mentioned factors contribute to the pathogenesis of cystogenesis is discussed briefly

1.5.1 Cell proliferation and apoptosis

Increase proliferation and apoptosis of renal tubular epithelial cells are a hallmark of cystogenesis (Evan, Gardner et al. 1979; Grantham, Geiser et al. 1987; Lanoix, D'Agati et al. 1996). The activity of tubular epithelial cell proliferation is associated with altered expression of various proto-oncogenes, proliferating cell nuclear antigen, growth factors and their receptors including c-myc, c-Ki-ras, c-fos and erbB-2, in cystic epithelial cells (Nadasdy, Laszik et al. 1995). Additionally, disturbance of calcium homeostasis and cAMP lead to disturbance of other signalling that involves in the regulation of cell proliferation, for instance B-Raf/MEK/ERK pathway (Yamaguchi, Hempson et al. 2006). Epidermal growth factors mediate tubular epithelial cells proliferation via a Ras/Raf-1/MEK/ERK pathway (Du and Wilson 1995; Yamaguchi, Hempson et al. 2006). Over-expression and mislocalization of EGFR to the apical membrane of tubular epithelial cells with mitogeneic concentration of EGFR in cystic fluid from patients with ADPKD have been discovered (Du and Wilson 1995; Orellana, Sweeney et al. 1995;

Zheleznova, Wilson et al. 2011). Taken together, an important role for epidermal growth factor in the mechanism of tubular epithelial cell proliferation has been reported (Du and Wilson 1995; Zheleznova, Wilson et al. 2011). Other factors that may directly stimulate renal epithelial cell proliferation including ATP, growth factors and cytokines (Sun, Zhou et al. 2011). Cyclic AMP and pro-proliferative cytokines have been found in cystic fluid (Gardner, Burnside et al. 1991; Merta, Tesar et al. 1997; Hanaoka and Guggino 2000).

Abnormalities of apoptosis have been demonstrated in various ADPKD models including: murine (Veis, Sorenson et al. 1993), rodent (Ecder, Melnikov et al. 2002), and human (Woo 1995). Of interest, over-expression of PC1 in MDCK cells induces resistance to apoptosis as well as spontaneous tubulogenesis (Boletta, Qian et al. 2000). Over-expression of c-myc, a proto-oncogen which is involved in cellular proliferation and apoptosis, in transgenic mice can induce ADPKD (Lanoix, D'Agati et al. 1996). Loss of the balance between intracellular pro- and anti-apoptotic members of a bcl-2 family was reported to be correlated with increased apoptosis in polycystic kidney of a Han:SPRD rat (Ecder, Melnikov et al. 2002).

1.5.2 Extracellular matrix (ECM) remodelling

The cystic kidney, a number of extracellular matrix alterations have been observed in human and animal models of ADPKD (Cuppage, Huseman et al. 1980; Wilson, Hreniuk et al. 1992; Calvet 1993; Schafer, Gretz et al. 1994). These changes include a thickness of basement membrane, alteration in integrin fibronectin receptor and heparan sulfate proteoglycan, increase expression of laminine (B1 and B2), and also type I and IV collagen (Wilson, Hreniuk et al. 1992; Calvet 1993; Ramasubbu, Gretz et al. 1998). Aberrant expression of laminin-332 and -5 might has regulatory roles in tubular epithelial cells proliferation and cyst formation (Joly, Berissi et al. 2006; Vijayakumar, Dang et al. 2014). A primary defect in ECM composition causes polycystic kidney in a PKD mouse model with hypomorphic mutation of laminin α5 (Shannon, Patton et al. 2006). These alterations in ECM composition may contribute to the pathogenesis of cystogenesis (Chang and Ong 2008).

1.5.3 Polarity

In the kidney of experimental models of ADPKD, changes in membrane polarity from basolateral membrane of tubular epithelial cells to the apical membrane have been reported previously (Chang and Ong 2008; Wilson 2011). These alterations include: EGFR/ErbB receptors and apical proteins including NKCC1 (Wilson, Devuyst et al. 2000; Wilson 2011). Apical location of E-cadherin could cause failure of basolateral

protein trafficking and account for their mislocation, for instance EGFR/ErbB receptors (Charron, Nakamura et al. 2000). Additionally, during tubulogenesis, defects in planar cell polarity or cell deviation orientation could play significant roles in the pathology of cystogenesis (Simons, Gloy et al. 2005; Fischer, Legue et al. 2006).

1.5.4 Fluid secretion

In patients with ADPKD, the size of the kidney is due to the accumulation of cystic fluid within tubular epithelial sacs (Grantham, Ye et al. 1995). In normal kidneys, more than 99% of the glomerular filtrate is reabsorbed by various tubular segments; therefore, the presence of this fluid is anomaly (Terryn, Ho et al. 2011). As renal cysts are separated from their tubular origin, the intra-cystic fluid most likely derives from trans-epithelial secretion, rather than from the glomerular filtrate (Grantham, Geiser et al. 1987; Terryn, Ho et al. 2011). Passive water and Na+ pumps (Na+/K+-ATPase) as well as transepithelial chloride secretion are the main mediator of tubular secretion (Grantham, Ye et al. 1995; Chang and Ong 2008). Mislocation of Na+/K+-ATPase in tubular epithelial cells of PKD kidneys have been observed; it is basolateral in the normal tubular epithelial cells, while in PKD, it is located on the apical membrane (Wilson, Sherwood et al. 1991). Additionally, cAMP may has regulatory roles in fluid transport (Mangoo-Karim, Uchic et al. 1989; Ye, Grant et al. 1992; Hovater, Olteanu et al. 2008).

1.5.5 Abnormality of renal primary cilia

ADPKD is classified as a ciliopathy disorder (Lee and Gleeson 2011). Primary cilia, a hair like sensory organelle that arises from centrioles, exists on the apical surface of the renal epithelial cells, beside almost all cells (Ong and Wheatley 2003; Deane and Ricardo 2007; Patel, Chowdhury et al. 2009). Structural or functional abnormality of primary cilia have been linked to the pathogenesis of cystogenesis (Chang and Ong 2008). PKD mice with mutations in Tg737, which is responsible for cystic kidney in that model, showed abnormal short primary cilia (Pazour, Dickert et al. 2000). Defect of ciliary proteins, for instance ciliary motor subunit (KIF3), in tubular epithelial cells produced cystic kidney in an animal model (Lin, Hiesberger et al. 2003; Ko and Park 2013). Primary cilia have a mechanosensory function in the renal epithelial cells; fluid flow induces bending of the primary cilia which leads to an increase in local calcium and further stimulates calcium release from intracellular stores (Praetorius and Spring 2001; Praetorius and Spring 2003; Delling, DeCaen et al. 2013; Chebib, Sussman et al. 2015). In the cystic epithelial cells, mutation of PC1 and PC2 causes disruption in mechanosensory function of the primary cilia (Nauli, Alenghat et al. 2003; Nauli,

Rossetti et al. 2006). This disturbance in calcium homeostasis leads to dysregulation of cAMP signalling, stimulation of proliferation and fluid secretion (Deane and Ricardo 2007; Chang and Ong 2008; Chebib, Sussman et al. 2015).

1.5.6 Signal pathways

It is hypothesised that *PKD1* and *PKD2* mutations result in disturbance in intracellular calcium, which in turn leads to accumulation of intercellular cAMP (Torres and Harris 2014). Cyclic AMP stimulates B-Raf/MEK/ERK pathways, cellular proliferation, and also chloride and fluid secretion though an action on CFTR. Polycystin mutations also lead to mTOR activation; and this can be further amplified by MAPK/ERK signalling on mTOR to promote cellular proliferation (Chapin and Caplan 2010). A part from mTOR signalling, other pathways regulated by PC1 and PC2 includes WNT signalling (Shillingford, Murcia et al. 2006; Lal, Song et al. 2008; Distefano, Boca et al. 2009) and JAK-STAT signalling pathways (Bhunia, Piontek et al. 2002).

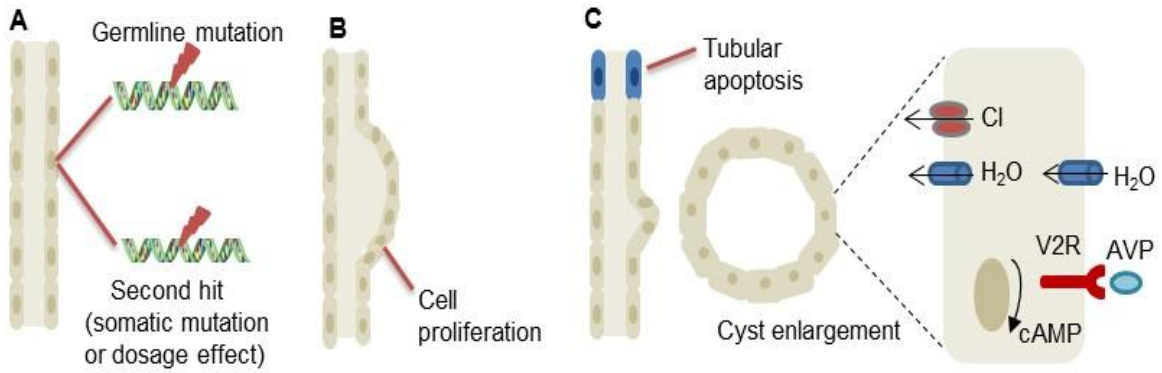

Figure 1.3. Renal tubular cyst formation and expansion

(**A**) Germline mutations of PKD genes with a second hit mechanism lead to (**B**) continuous cellular division and abnormal planer cell polarity that lead to radial expansion of renal tubular walls, (**C**) cyst formation and separation from their tubules and (**D**) fluid fill the tubular sac by involving of cAMP mediate chloride influx through arginine vasopressin receptors (AVP) in renal tubular epithelial cells (Wilson 2004; Hassane, Leonhard et al. 2010). The figure was redrawn from Grantham et al. (Grantham and Torres 2016) with permission.

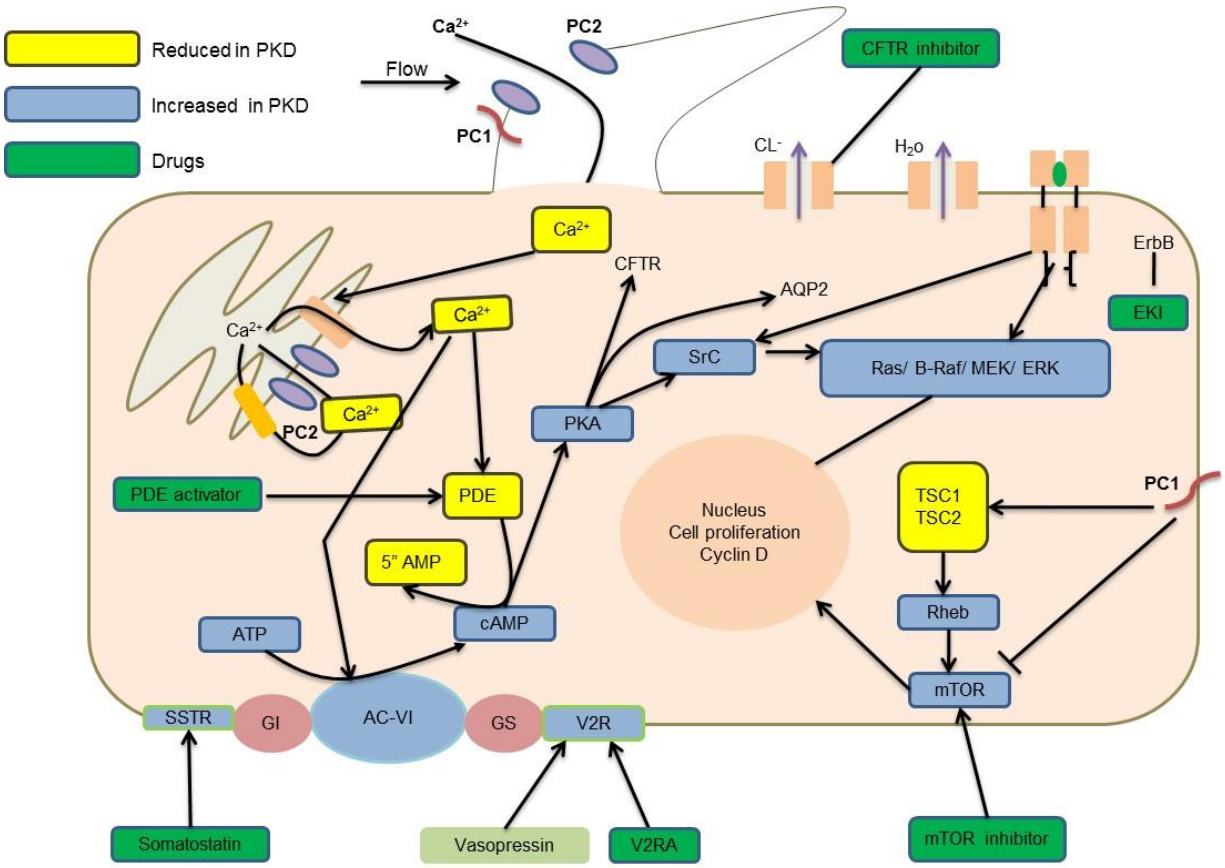

Figure 1.4. Signalling pathway abnormalities in ADPKD and molecular targets

Abbreviations are: mTOR; mammalian target of rapamycin, SSTR; Somatostatin receptor, V2R; Vasopressin V2 receptor, ATP; Adenosine triphosphate, AQP2; Aquaporin2, CFTR; Cystic Fibrosis Transmembrane Conductance regulator, PDE; Phosphodiesterase. The figure was redrawn from Sun et al. (Sun, Zhou et al. 2011) with permission.

1.6 Clinical features of ADPKD

The clinical manifestations of ADPKD can be usefully classified into cystic and non-cystic. Regarding the cystic manifestations, the most frequent extra renal manifestation is liver cysts (Wolyniec, Jankowska et al. 2008). Other sites of cyst formation are the pancreas (5-10%), spleen, arachnoid and thyroid (Boucher and Sandford 2004).

Among the non-cystic manifestations, hypertension (HTN) occurs in up to 80% of patients and may be the earliest sign of ADPKD in childhood (Helal, Reed et al. 2012). Decreases in kidney blood flow and alterations in renal sodium handling usually accompany the onset of HTN (Torres, Harris et al. 2007). In polycystic kidneys, cyst expansion leads to stretching of the vascular tree resulting in activation of the renin-angiotensin system (Gabow, Chapman et al. 1990).

The most frequent symptom of ADPKD is flank pain, which occurs in approximately 60% of the cases and it is usually caused by cyst haemorrhage, infection of the cyst or urinary tract, kidney stones or in frequently tumours (Torres, Harris et al. 2007). Macroscopic haematuria is another common symptom of ADPKD and occurs in around 40% of the cases (Gabow, Duley et al. 1992). Additionally, nephrolithiasis occurs in 20% of ADPKD patients; uric acid or calcium oxalate is the most common type of ADPKD stones (Torres, Harris et al. 2007). In approximately 25% of the cases, heart valve defects occur concomitantly with ADPKD (Ecder and Schrier 2009). Around 16% of ADPKD patients with a positive family history of the disease and 6% of those with a negative history of the disease have intracranial vascular aneurysms (Torres, Harris et al. 2007; Tan, Blumenfeld et al. 2011). An increased incidence of abdominal hernias has been described in ADPKD (Boucher and Sandford 2004). The urinary concentration ability of the kidneys is impaired early in the course of disease (Torres, Bankir et al. 2009).

Cyst expansion leads gradually to loss of renal function and chronic kidney disease (CKD). CKD is defined as renal damage by function or structural abnormalities that cause renal damage or by eGFR < 60 ml/min/1.73^{2}m with or without renal damage for more than 3 months (Levey, Eckardt et al. 2005). According to K/DOQI guidelines (Kidney Disease Quality Outcome Initiative), patients with CKD are classified into 5 stages according to their eGFR measurement (**Table 1.1**) (Levey, Coresh et al. 2003).

Table 1.1. Classification of CKD

Stages	eGFR	Description
1	≥ 90 ml/min	Kidney damage with normal or > eGFR
2	60–89 ml/min	Kidney damage with mild decreased eGFR
3	30–59 ml/min	Moderate decreased eGFR
4	15–29 ml/min	Severely decreased eGFR
5	< 15 ml/min	Renal failure

The figure summarized stages of CKD according to eGFR. Stage 3 is further classified into 2 stages; 3A with eGFR 45–59 ml/min and 3B with eGFR 30–44 ml/min.

1.7 Diagnosis and Screening

In kidney disease, eGFR is the standard measure of renal function. However, eGFR may give misleading information about cystic disease burden since renal function can be maintained through compensatory hyperfiltration or serum creatinine altered by changes in the tubular handling of creatinine (Ruggenenti, Gaspari et al. 2012).

1.7.1 Radiological imaging

Because of accessibility, low cost and reproducibility, ultrasonography (US) is the most commonly used radiological modality for diagnosing of ADPKD (Wolyniec, Jankowska et al. 2008). Age-banded criteria for US based diagnosis in individuals with a positive family history of ADPKD have been published and revised (**Table 1.2**) (Pei, Obaji et al. 2009).

Table 1.2. Unified ultrasonography criteria for diagnosis of ADPKD

Age (years)	Number of cysts
15-39	3 or > unilateral or bilateral kidney cysts both kidneys
40-59	2 or > in each kidney
> 60	At least 4 in each kidney

The table was reproduced from Pei et al. (Pei, Obaji et al. 2009).

Other radiological modalities used for ADPKD diagnosis include MR and CT imaging. These techniques are more sensitive than US especially in patients younger than 20 (Wolyniec, Jankowska et al. 2008). New MRI criteria for people younger than 30 years of age have been reported (Pei, Hwang et al. 2015): a total of more than 10 cysts in each kidney provide 100% of sensitivity and specificity (Pei, Hwang et al. 2015). However, the higher costs of CT and MRI restrict their use to diagnosis of uncertain cases (Wolyniec, Jankowska et al. 2008).

Total kidney volume (TKV) based on MRI or CT scan is currently considered the most sensitive prognostic marker of disease progression (Wolyniec, Jankowska et al. 2008; Grantham and Torres 2016). Serial radiological imaging can also provide a more sensitive measure of disease progression especially when eGFR is preserved (Helal, Reed et al. 2012). Using MRI based techniques, the error in renal volume measurement is less than 5% (Wolyniec, Jankowska et al. 2008). Different studies have described the relationship between TKV and eGFR as an inverse relationship in the late stages of the disease (Helal, Reed et al. 2012). However, MR imaging is not currently standard clinical practice and TKV is most often used in clinical research (Wolyniec, Jankowska et al. 2008). A decline in renal blood flow (RBF) can be a very early sign of ADPKD disease preceding the onset of hypertension (Helal, Reed et al. 2012). The CRISP study concluded that, RBF is an independent predictor factor for PKD progression (Torres, King et al. 2007). Currently, the use of RBF is only used as a research tool (Wolyniec, Jankowska et al. 2008; Helal, Reed et al. 2012).

1.7.2 Genetic testing

There are two available techniques for DNA analysis, genetic linkage and direct mutation analysis (Pei and Watnick 2010). Linkage studies require several affected and non-affected family' members to be tested. Mutation analysis of *PKD1* has been complicated by its size, complex structure and duplication into 6 highly homologous pseudogenes (97.76% sequence identity) on chromosome 16 (Bogdanova, Markoff et al. 2001; Rossetti, Chauveau et al. 2002; Rossetti, Consugar et al. 2007; Rossetti, Hopp et al. 2012). Mutation analysis can identify a causative mutation in 90% of cases (Rossetti, Consugar et al. 2007; Trujillano, Bullich et al. 2014). The recent development of next generation sequencing may reduce the high cost of testing (Eisenberger, Decker et al. 2015). In clinical practice, the current policy is to request genetic testing when the diagnosis is uncertain after radiological imaging for instance in younger individuals (less than 40 years) who could be potential kidney donors (Wolyniec, Jankowska et al. 2008; Simms, Travis et al. 2015).

1.8 Natural history of ADPKD

The natural course of ADPKD is hugely variable and is associated with intrafamilial and interfamilial variability, even between patients with identical mutations (Torres, Harris et al. 2007; Chang and Ong 2008; Thong and Ong 2013). The typical course is of adult-onset disease with ESRD (defined as the requirement of dialysis or transplantation) around the age of sixty, however, a proportion of patients has adequate renal function around the age of ninety, whereas others present with renal impairment from childhood (Harris and Rossetti 2010; Cornec-Le Gall, Audrezet et al. 2013; Schrier, Brosnahan et al. 2014). Disease progression may be defined as onset of hypertension at age less than <18 years, development of stage 3 CKD at age less than < 40 years, onset of ESRD at age less than 55 years, total kidney volume more than that expected for a given age, or presence of multiple complications (Schrier, Brosnahan et al. 2014).

The rate of disease progression to ESRD depends on the interaction between genetic and non-genetic factors (Choukroun, Itakura et al. 1995). Reported risk factors for ESRD are *PKD1* mutation, diagnosis at an early age, male gender, presence of haematuria, proteinuria or hypertension recognized under 35 years, left ventricular hypertrophy and liver cysts in young female patients (Gabow, Johnson et al. 1992; Wolyniec, Jankowska et al. 2008; Helal, Reed et al. 2012). Other modifiable factors include higher sodium excretion, higher urine osmolality and lower serum HDL have

been reported as potential risk factors for disease progression (Torres, Grantham et al. 2011).

The CRISP study which aimed to discover reliable early measures of disease progression in ADPKD enrolled 27 *PKD2* and 156 *PKD1* patients who were observed annually by MRI and measured eGFR initially over 3 years (Chapman, Guay-Woodford et al. 2003). They found that *PKD1* patients had larger kidneys and greater cyst number compared to *PKD2* patients; TKV increased by approximately 5.3% per year over the observation period (Chapman, Guay-Woodford et al. 2003; Chapman 2008; Pei and Watnick 2010). It has been shown that patients with PKD1 truncating mutations have the worst outcome followed by PKD1 non-truncating mutations and PKD2 (Cornec-Le Gall, Audrezet et al. 2013; Ong, Devuyst et al. 2015; Hwang, Conklin et al. 2016).

Recently, 2 risk prediction models have been proposed for ADPKD disease progression: the Mayo imaging classification and the PROPKD score (Mao, Chong et al. 2016). The Mayo classification was developed from serial measurements of height-adjusted TKV (for age) and 3 or more eGFR readings over > 6 months in a retrospective study of a single centre cohort of 590 patients and was further validated in the independent CRISP cohort of 173 patients (Irazabal, Rangel et al. 2015). Based on their imaging, patients could be divided into 2 classes; class 1 (typical radiological features of ADPKD = 91.2%) and class 2 (atypical radiological features of ADPKD = 8.8%). Class1 patients were further divided into 5 subclasses (A-E), according to their rate of kidney growth which correlated with the rate of eGFR decline. Patients in class 1A and 1B had slowly progressive disease, while patients with class 1C-E had rapidly progressive disease (> 3 ml/min per year). Using this scoring system improved the ROC curve of disease progression for patients with eGFR > 60 ml/min from 86.4 to 90.1%, and in the patients with eGFR < 60 ml/min from 90 to 93.4%.

Regarding PROPKD (Cornec-Le Gall, Audrezet et al. 2016), a prognostic model was developed to predict ADPKD progression based on a multivariate survival analysis of genetic and clinical data of the GenKyst cohort (n = 1341). The score ranged from 0 to 9 and was calculated as follows: 1 point for being male; 2 points for non-truncating *PKD1* mutation; 4 points for truncating *PKD1* mutation; among patients younger than 35 years, 2 points for hypertension and 2 points for early urological symptoms. Based on these factors, patients were classified into 3 risk group's i.e. low risk (0-3 points), intermediate risk (4-6 points) and high risk (7-9 points). These models provide an evidence-based framework for stratifying patients into different risk groups for early, intermediate or late disease progression. However, they may still be insufficiently sensitive for predicting individual prognosis given the significant overlap between

groups (Mao, Chong et al. 2016). Additionally, access to genotype and radiological imaging (CT scan and MRI) is still restricted to few clinical indications outside the research setting (Rangan, Tchan et al. 2016).

Several serum and urinary biomarkers of disease have been reported as potential diagnostic biomarker for ADPKD progression (**Table 1.3**). These include serum copeptin which is a surrogate marker of endogenous vasopressin (Bolignano, Cabassi et al. 2014). Serum copeptin has been reported as a potential biomarker for the disease progression (Meijer, Bakker et al. 2011; Boertien, Meijer et al. 2012; Boertien, Meijer et al. 2013; Nakajima, Lu et al. 2015). However, this biomarker is still lack the specificity for ADPKD; it displayed considerable overlap with non-ADPKD patients with CKD (Corradi, Martino et al. 2016). Contradictory results about the diagnostic value of neutrophil gelatinase-associated lipocalin (NGAL) for the disease progression were reported in the literature (Meijer, Boertien et al. 2010; Parikh, Dahl et al. 2012; Kawano, Muto et al. 2015). Additionally, serum and urine levels of NGAL reflect the severity of kidney function impairment in patients with various aetiologies of CKD (Bolignano, Lacquaniti et al. 2008; Bolignano, Lacquaniti et al. 2009; Rubinstein, Pitashny et al. 2010; Haase, Haase-Fielitz et al. 2011; Smith, Lee et al. 2013; Soltysiak, Skowronska et al. 2014; Rhee, Shin et al. 2015). Urine fetuin-A, a negative acute phase protein, is another promising biomarker for ADPKD progression, with 94% sensitivity and 60% specificity at a cut-of value of 12.2 µg/mmol fetuin-A /creatinine to differentiate healthy controls from patients with ADPD (Piazzon, Bernet et al. 2015). On the other hand, the potential role of urine fetuin-A as a potential novel biomarker has been reported for acute kidney injury (Zhou, Pisitkun et al. 2006) and type 2 diabetic nephropathy (Inoue, Wada et al. 2013). **Table 1.3** summarises a number of biomarkers which has been reported as potential biomarker for ADPKD progression, however, none of these biomarkers have been further validated to be applied for clinical practice.

Table 1.3. Biomarker predictors associated with ADPKD progression

Biomarker	Study details	Method of statistically analysis	Conclusion	Reference
Copeptin	**1**. A cross sectional study in ADPKD patients (n = 102) **2**. A prospective cohort in ADPKD patients (n = 79) with 3.3 years' follow-up **3**. A longitudinal observational study of ADPKD patients (n = 241) with 8.5 years' follow-up **4**. Cross sectional study of ADPKD patients (n = 50)	**1**. Regression analysis **2**. ROC curve, sensitivity and specificity were not measured.	Blood and urine levels of copeptin associated with ADPKD severity.	**1.** (Meijer, Bakker et al. 2011) **2.** (Boertien, Meijer et al. 2012) **3.** (Boertien, Meijer et al. 2013) **4.** (Nakajima, Lu et al. 2015)
Plasma copeptin	A case-control study in ADPKD patients (n = 112) and non-ADPKD CKD patients (n = 112)	**1**. Spearman correlation **2**. ROC curve, sensitivity and specificity were not measured.	Copeptin is associated with renal function rather than the underlying aetiology.	(Corradi, Martino et al. 2016)
Urinary NGAL, β2-microglobulin, H-FABP, KIM-1 and MCP-1	A cross sectional study in ADPKD patients (n = 102) and healthy controls (n = 102)	**1**. Spearman correlation **2**. Regression analysis, sensitivity and specificity were not measured.	All the examined biomarkers, especially NGAL might be of a diagnostic value for assessment of ADPKD severity.	(Meijer, Boertien et al. 2010)
Urinary NGAL and IL-18	A prospective cohort in ADPKD patients (n = 107) with 3 years' follow-up, cystic fluid and cystic renal tissue of Han:SPRD rat model of polycystic disease	**1**. Annual % changes in TKV, NGAL and IL-18 **2**. ROC curve, sensitivity and specificity were not measured.	Changes in urinary levels of NGAL and IL-18 did not correlate with change in TKV.	(Parikh, Dahl et al. 2012)
Urinary angiotensinogen	A case-control study in ADPKD patients (n = 233)	**1**. Regression analysis **2**. ROC curve, sensitivity and specificity were not measured.	AGT/Cr was significantly increased in the patients with CKD stages 3 and 4.	(Park, Kang et al. 2015)
Urinary NAG, β2-microglobulin and KIM-1	A prospective cohort in ADPKD patients (n = 270 with 1 year' follow-up	Regression analysis and ROC curves	Among the examined biomarker, only NAG showed a significant ability to discriminate patients with a stable disease from those with a progressive disease (AUC = 0.79). But NAG failed to predict disease progression after a year.	(Park, Hwang et al. 2012)

Urinary PKD1N, PKD1C, PKD2C, L-FABP, LDH, AQP2, vWF, IL-18, IL-8, 8-OHdG, 8-Isoprostane, M-CSF, VEGF, IFNAE2, TFF1, TFF2, angiotensinogen, TFF3, PEPI, NGAL, MCP-1, vasopressin, aldosterone, HGF, cAMP, lysozyme and ceruloplasmin	A cross sectional study in ADPKD patients (n = 23). and healthy controls (n = 6) Urine sample from DBA/2FG-pcy mouse	Not mentioned	NGAL, MCP-1 and M-CSF are potential diagnostic biomarkers.	(Kawano, Muto et al. 2015)
MCP-1	Mice model of ADPKD (CD1(pcy/pcy) (pcy))	One-way ANOVA	MCP-1 is an early indicator of ADPKD progression.	(Kirby, Stepanek et al. 2014)
Serum angiopoietin 1, angiopoietin 2 and VEGF	A cross sectional study in young ADPKD patients (n = 71) and young patients with DM (n = 10)	Regression analysis	Serum levels of VEGF were negatively correlated with creatinine clearance. Angiopoietin level was correlated with urinary protein.	(Reed, Masoumi et al. 2011)
Urine fetuin-A	A cross sectional study in ADPKD patients (n = 66) and healthy controls (n = 17) Cystic renal tissues of Bicc1 and *Pkd1* mouse tissues	Regression analysis, ROC curve, sensitivity and specificity	Upregulated in Bicc1 and *Pkd1* mouse models of ADPKD. It is a sensitive diagnostic biomarker for ADPKD progression.	(Piazzon, Bernet et al. 2015)

Abbreviations are: NGAL; neutrophil gelatinase-associated lipocalin, TKV; total kidney volume, NAG; N-acetyl-β-D glucosaminidase, KIM-1; kidney injury molecule-1, H-FABP; heart-type fatty acid binding protein, VEGF; vascular endothelial growth factor, vWF; von Willebrand factor, IL-8; Interleukin 8, MCP-1; monocyte chemoattractant protein-1, LDH; lactate dehydrogenase, L-FABP; liver-type fatty acid-binding protein, IL-8; Interleukin 18, 8-OHdG; 8-hydroxydeoxyguanosine, TTF; trefoil factor family.

1.9 Treatment of ADPKD

1.9.1 General principles

Current available management is aimed at treating or preventing disease complications, such as, pain, haemorrhage and treatment of urinary tract or cyst infections (Masoumi, Reed-Gitomer et al. 2008). The HALT-PKD (study A) was a randomized control trial which aimed to establish the advantages of ACE inhibitors and an ARB as combination therapy over ACEI alone in slowing disease progression in the patients with eGFR > 60 ml/min (Chapman, Torres et al. 2010). It also sought to determine an appropriate blood pressure target that effectively slows the progression of ADPKD, by comparing standard (120/70 to 130/80 mm Hg) against very low blood pressure control (95/60 to 110/75 mmHg) (Chapman, Torres et al. 2010). The outcomes measured in this study were changes in eGFR and TKV. The study concluded that, combination of ACE inhibitors and an ARB did not significantly change the rate of TKV increase, however, a lower blood pressure target led to a slower rate in TKV growth, decreased urinary albumin excretion and reduced left ventricular mass (Schrier, Abebe et al. 2014; Torres, Abebe et al. 2014).

1.9.2 Novel therapies

The discovery of novel therapies for ADPKD has been based on a clearer understanding of the major pathophysiological mechanisms and signalling pathways implicated in the pathogenesis of ADPKD (Chang and Ong 2012; Mahnensmith 2014). Currently, the major targets in developing new drugs are based on decreasing cAMP levels, inhibiting proliferation of cyst epithelial cells and decreasing fluid secretion into cysts (Chang and Ong 2012).

Vasopressin antagonists

Cyclic AMP production is stimulated by vasopressin through V2 receptors in renal collecting ducts, the principle location of cyst growth (Chang and Ong 2012). This was the foundation for the TEMPO 3:4 trial, which tested the efficacy of a vasopressin antagonist (tolvaptan) compared to placebo in 1445 patients over 3 years. The increase in TKV was 2.8% per year in the tolvaptan group versus 5.5% in the placebo group. Moreover, there was a lower rate of decline in renal function in the tolvaptan group (P value < 0.001) (Torres, Chapman et al. 2012). However, the rate of discontinuation was higher in the tolvaptan group than other group due to its side effects including thirst,

polyuria and abnormal liver function tests (Black and Sutton 2013). So far, tolvaptan has been approved by regulatory agencies in the UK, Japan, Canada, Europe and South Korea (Mao, Chong et al. 2016). The European Medicines Agency (EMA) recommended the use of tolvaptan for treatment of ADPKD patients with stage 1-3 CKD with evidence of rapid disease progression (Gansevoort, Arici et al. 2016; Mao, Chong et al. 2016; Ong A 2016).

The mammalian target of rapamycin inhibitors (mTOR inhibitors)

In experimental models of ADPKD, activation of mTOR is a consistent finding linked to de-differentiation of the tubular epithelial phenotype (Wuthrich and Serra 2009). The SIRENA study, a small randomized study, was the first to report the short-term effect of mTOR inhibitors on ADPKD progression (Perico, Antiga et al. 2010). 15 participants were randomized into 2 groups: sirolimus or conventional management alone for 6 months. The results of this study showed no significant difference in TKV between the two arms (P value = 0.45) (Perico, Antiga et al. 2010). In a second trial, 100 ADPKD patients were randomized to receive either sirolimus or conventional therapy for 18 months. These study results were similar to those of the SIRENA study (Serra, Poster et al. 2010). In a third trial, 433 ADPKD patients were randomized to receive either everolimus or conventional therapy over 2 years (Walz, Budde et al. 2010). The most remarkable result of this study was the dissociation of the everolimus effect on TKV and eGFR: everolimus slowed the rate of kidney volume growth but did not prevent the decline in eGFR (Walz, Budde et al. 2010; Chang and Ong 2012). There was a high patient dropout rate in this trial due to treatment related adverse effects.

Somatostatin analogues

Since cyclic AMP is known to stimulate cystic cell proliferation and enhance fluid secretion in ADPKD, other agents that reduce cellular cAMP levels such as somatostatin analogues may be effective treatments to retard cyst expansion (Chang and Ong 2012). The effects of octreotide on ADPKD and polycystic liver disease (PLD) were tested in a double blind randomized placebo-controlled study in 42 patients over a year. TKV and total liver volume (TLV) were measured by MRI. Liver volume changes were considerably lower in octreotide than placebo group, while TKV was significantly increased in the control group compared to the octreotide group P value = 0.045 (Hogan, Masyuk et al. 2010). This result was confirmed in a second study of 54 PLD and ADPKD patients randomized to receive lanreotide or placebo for 6 months. Hepatic volume decreased by 2.9% in the lanreotide group compared to placebo (van

Keimpema, Nevens et al. 2009). In the same way, TKV increased in the control group by 3.4%; whereas it decreased by 1.5% in the lanreotide group (Chang and Ong 2012).

1.10 The rationale for identifying prognostic disease biomarkers

The introduction of a potent novel therapy (tolvaptan), which can modify the natural history of disease but has significant side effects, is expensive and requires life-long administration, has made the search for new predictive or prognostic disease biomarkers more urgent. Biomarkers that can help select patients that are at highest risk of disease progression, predict or monitor treatment response and toxicity will be of significant clinical utility.

1.11 Biomarkers of kidney disease

Since the clinical course of ADPKD is so variable, a useful biological biomarker needs to distinguish patients with good or poor prognosis (Helal, Reed et al. 2012). The first stage is biomarker discovery while in the second stage, biomarker validation proceeds by defining its sensitivity and specificity. The final step is implementation, by defining the clinical assay which will be used to introduce a valid biomarker into clinical practice (Pisitkun, Johnstone et al. 2006).

1.11.1 Characteristics of a perfect biomarker of kidney disease

A biological biomarker can be defined as a biological substance that indicates a physiological or pathological process as well as reflect the pharmacological response to various medications. Moreover, this substance should be measurable in an objective manner (Hewitt, Dear et al. 2004). The discovery of an ideal biomarker is dependent on the nature of the disease. However, there are general features which are essential for any biological biomarker (Edelstein 2011). An ideal biomarker should be inexpensive, accurate and rapidly assayed (Hewitt, Dear et al. 2004; Edelstein 2011). It should be non-invasive, obtained from an accessible source, for instance, urine or blood. In addition, the sensitivity and specificity of the biomarker should be high (Edelstein 2011).

1.11.2 Source of biomarkers

Renal biopsy tissue, blood and urine are the main sources of potential biomarkers in renal diseases. Renal biopsy is not routinely performed in patients with ADPKD (Hiura, Yamazaki et al. 2006; Peces, Martinez-Ara et al. 2011; Hogan, Mocanu et al. 2016). Compared to plasma, urine as a source for potential biomarkers discovery has a number of advantages (Li 2015). Firstly, urine reflects the accumulation of pathophysiological changes in the body whereas strict homeostatic regulations control blood composition more acutely (Gao 2013; Li 2015; Li and Gao 2016). Secondly, urine can be collected more frequently and in a larger quantity compared to blood sampling (Li 2015). Additionally, urine proteomes may be more stable; proteolytic activity occurs prior to micturition while protease activity may occur at the time of blood collection (Yi, Kim et al. 2007; Decramer, Gonzalez de Peredo et al. 2008; Li 2015).

Another promising source of biomarkers is exosomes, small vesicles (40-100 nm) that originate from the endosomal system (Hoorn, Pisitkun et al. 2005; Raposo and Stoorvogel 2013). The field of exosome research originated with a hypothesis that exosomes serve as a waste disposal system from rat and sheep cells (Harding, Heuser et al. 1983; Pan and Johnstone 1983; Pan, Teng et al. 1985). Since then, researchers have reported excretion of exosomes by many cell types including stem cells (Lopez-Verrilli, Caviedes et al. 2016); skeletal muscle cells (Choi, Yoon et al. 2016); cancer cells (Kruger, Abd Elmageed et al. 2014); cardiomyocytes (Sluijter, Verhage et al. 2014); hepatocytes (Nojima, Freeman et al. 2016); renal epithelial cells (Pisitkun, Shen et al. 2004); and neuronal tissue (Kalani and Tyagi 2015).

Exosomes are believed to originate from multivesicular bodies (MVB) through the inward budding of a section of the endosomal membranes; afterwards, the MVB fuse with the plasma membrane by exocytosis releasing its contents into the extracellular space (**Figure 1.5A**) (Kowal, Tkach et al. 2014). Exosomes contain a specific set of lipid, nucleic acid and proteins, and are surrounded by a lipid bilayer membrane that protects their contents from enzymatic degradation (**Figure 1.5B**) (Enderle, Spiel et al. 2015). Exosomes have been found to contain DNA, mRNA, microRNAs and other small RNAs (Valadi, Ekstrom et al. 2007; Nolte-'t Hoen, Buermans et al. 2012; Thakur, Zhang et al. 2014; Jin, Chen et al. 2016). Previous publications reported enrichment of exosomes with phosphatidylserine, sphingomyelin, ceramide and cholesterol (Matsuo, Chevallier et al. 2004; Kowal, Tkach et al. 2014). Additionally, exosomes contain a specific set of proteins derived from an endosomal compartment, cytosol and plasma membrane, but a limited amount of proteins from the nucleus, Golgi, mitochondria and endoplasmic reticulum (Bobrie, Colombo et al. 2011; Thery 2011). For instance, this set

of proteins includes: tetraspanins (CD9, CD63, CD81 and CD82); MVB biogenesis (Alix and tumour susceptibility gene-101 (TSG-101)); receptors (tumour necrosis factor receptor-1); antigen presentation molecules (MHC1 and MHC2); heat shock proteins (HSP20, HSP60, Hsp700 and Hsp90); cell structure, adhesion and motility proteins (Integrin, tubulin, myosin and actins); signalling proteins (kinases and heterotrimeric G-proteins) (Keller, Sanderson et al. 2006; Urbanelli, Magini et al. 2013). Of interest, important roles for exosomes in physiological and pathological conditions have been reported in intracellular communication (Simons and Raposo 2009); immune response (Raposo, Nijman et al. 1996); lactation (Admyre, Johansson et al. 2007); neurodegenerative disease (Vella, Sharples et al. 2008); and malignancy (Schorey and Bhatnagar 2008). In addition, exosomes have been utilized as an endogenous therapeutic vehicle for immunotherapy and gene therapy (Viaud, Thery et al. 2010; El-Andaloussi, Lee et al. 2012; O'Loughlin, Woffindale et al. 2012; Zhou, Zhou et al. 2016).

Exosomes reflect the pathophysiological processes which occur in their cells of origin (Moon, You et al. 2011), and exosomes have been found in various biofluids including urine (Pisitkun, Shen et al. 2004; Gonzales, Zhou et al. 2010); cerebrospinal fluid (Street, Barran et al. 2012); and plasma (Caby, Lankar et al. 2005). Therefore, there has been a growing interest in studying exosomes as a promising source of diagnostic biomarkers in various clinical conditions. Approximately 20 proteins of the isolated exosomes were previously recognized to have roles in different renal diseases, for instance ADPKD, familial renal hypomagnesemia and Bartter syndrome (Pisitkun, Shen et al. 2004; Dimov, Jankovic Velickovic et al. 2009). Transcriptomics profiling of exosomes from patients with tumours revealed that exosomes carry specific genetic information of that tumour (Nilsson, Skog et al. 2009; Record, Subra et al. 2011).

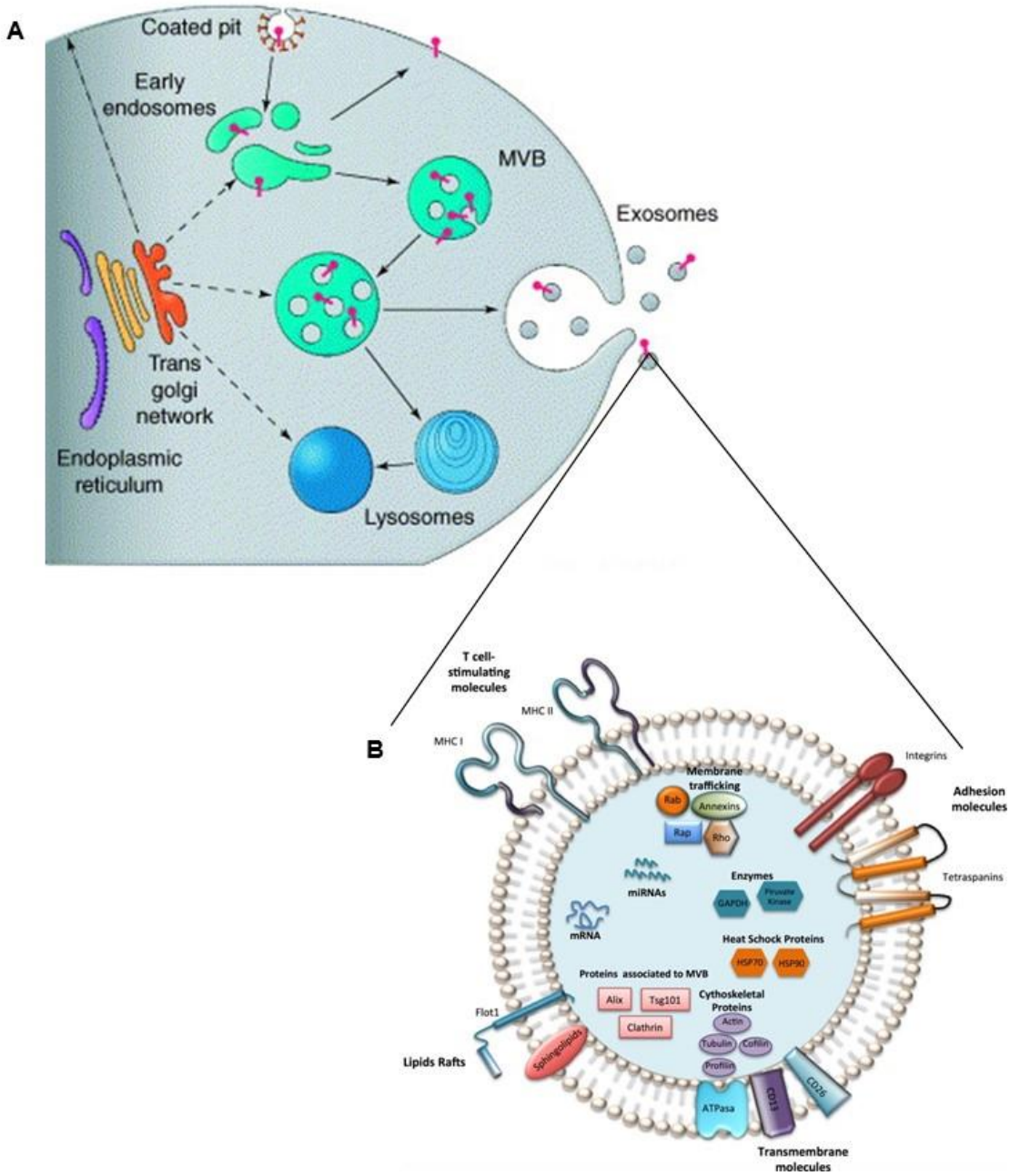

Figure 1.5. Exosomes biogenesis and composition

(**A**) The figure depicting intracellular mechanism of biogenesis and secretion of exosomes into extracellular space. The figure was modified from Fevrier et al. (Fevrier and Raposo 2004) with permission. (**B**) The figure shows molecular composition of exosomes (Merino-Gonzalez, Zuniga et al. 2016).

1.11.3 Experimental biomarkers in ADPKD

Proteomics

Urinary proteomics analysis examines the expression of multiple proteins simultaneously (Fliser, Novak et al. 2007). A number of studies have analysed urine proteomics of patients with ADPKD (**Table 1.4**). In one study, high molecular weight proteins > 10 kDa from patients with ADPKD (n = 30) were compared to healthy individuals (n = 30) (Bakun, Niemczyk et al. 2012). The authors found 155 proteins that were significantly altered in ADPKD urine including complement proteins and growth factors (Bakun, Niemczyk et al. 2012). A second study examined low molecular weight proteins ≤ 15 kDa in ADPKD patients (n = 41) and healthy controls (n = 86); 38 proteins were found to be significantly altered in ADPKD (Kistler, Mischak et al. 2009). These results were further validated in samples from the CRISP cohort (n = 251), the SUISSE ADPKD study (n = 68) and healthy controls (n = 86) (Kistler, Serra et al. 2013) using capillary electrophoresis coupled to mass spectrometry. That study also sought to develop a diagnostic biomarker model for ADPKD severity and progression using urine proteomics as well as a diagnostic test for undiagnosed young individuals, especially those with a *PKD2* genotype (Kistler, Serra et al. 2013). Based on 142 most consistent dysregulated peptides in urine samples from ADPKD patients, the model showed a significant correlation with height adjusted total kidney volume provided an 84.5% sensitivity and 94.2% specificity. However, the performance of this model showed a low sensitivity in young patients as well as patients with *PKD2* mutation, and also reduced specificity in older patients. Although interesting, these findings will require validation in other cohorts using an easier technique.

Table 1.4. Urine proteomics of potential diagnostic value in ADPKD

Study design	Experimental design	Discovered proteome	Validated proteome	Significant protein	Reference
Prospective cohort study of ADPKD patients (n = 30) and healthy controls (n = 30)	Proteome fraction with molecular weight > 10 kDa	155 proteins identified using MS in pooled samples	17 proteins were validated using MRM and Itraq.	8 proteins	(Bakun, Niemczyk et al. 2012)
Prospective cohort study of ADPKD patients (n = 17) and healthy controls (n = 86)	Proteome fraction with molecular weight <15 kDa	197 proteins using CE-MS	CE-MS repeated 10 times to validate the discovery phase and proteins found in at least 7 of 10 repeats were accepted.	38 proteins	(Kistler, Mischak et al. 2009)
A multicentre cohort Study	CE-MS	657 peptides	/	142 peptides	(Kistler, Serra et al. 2013)
Prospective cohort study of ADPKD patients (n = 6) and healthy controls (n = 6)	A labelled approach followed by a label-free approach of urine exosomes	30 proteins	periplakin, envoplakin, villin-1 and complement C3 and C9	Plakins and Complement	(Salih, Demmers et al. 2016)

Abbreviation is: CE-MS; Capillary electrophoresis coupled online to mass spectrometry, MRM; Multiple Reaction Monitoring, iTRAQ; isobaric tags for relative and absolute quantitation.

Chemokines

Chemokines refer to small heparin binding proteins (5-20 kDa) which are a specific category of chemotactic cytokines that act as chemical messengers of the immune system (Van Coillie, Van Damme et al. 1999). Approximately 50 chemokines have been identified so far with 20-70% homology of their amino acid sequence (Van Coillie, Van Damme et al. 1999; Baggiolini and Loetscher 2000). The principle functions of chemokines are to stimulate leukocyte recruitment and activation but they also play roles in regulating apoptosis and proliferation (Adams and Lloyd 1997). Moreover, chemokines can regulate the functions of other cell types such as fibroblasts and endothelial cells (Anders, Vielhauer et al. 2003; Segerer 2003). The chemokines family are divided into 4 subfamilies including CXC, CX, C and CX_3C (**Table 1.5**) (Comerford and McColl 2011). Chemokines act by binding to special transmembrane receptors (heterotrimeric G protein-coupled receptors) (**Figure 1.6**) which are classified according to their chemokine class, namely, CR, CXCR, CX3R, C and CCR (**Table 1.5**) (Anders, Vielhauer et al. 2003; Moser, Wolf et al. 2004). A variety of cells produce chemokines including endothelial, epithelial, fibroblast cells and different renal cells (Stahl 1997). Chemokine levels are increased in various diseases and can be used in the monitoring the activity of inflammatory processes and related disease progression (Sullivan, Cutilli et al. 2000).

The first indication of the possible role of chemokines in renal disease came from a rat model of proliferative glomerulonephritis. Using northern blot analysis and immunohistochemistry, no mRNA levels for MCP-1 were detectable in control glomeruli, but increased markedly 30 minutes after the induction of the nephritis (Stahl, Thaiss et al. 1993). Interestingly, chemokines can be detected in urinary samples when they are undetectable in serum (Wong and Singh 2001; Zheng, Wolfe et al. 2003). High MCP-1 expression in kidney tissue has been reported in various aetiologies of CKD (Prodjosudjadi, Gerritsma et al. 1995), and urinary MCP-1 levels have been shown to be altered in a number of kidney diseases including diabetic nephropathy (Tam, Riser et al. 2009; Titan, Vieira et al. 2012); chronic glomerulonephritis (Bobkova, Chebotareva et al. 2006); IgA nephropathy (Stangou, Alexopoulos et al. 2009); ANCA associated renal vasculitis (Tam, Sanders et al. 2004); lupus nephritis (Singh, Usha et al. 2012); and ADPKD (Zheng, Wolfe et al. 2003). Based on the principle functions of chemokines, identification of chemokine receptor antagonists are attractive drug targets in clinical research, and blockade of MCP-1 activity has shown to improve disease outcome in experimental models (Wells, Power et al. 1998; Kim and Tam 2011).

Although ADPKD is not considered an inflammatory disease, interstitial infiltration, inflammation and fibrosis have been observed in the early stages of cyst growth in human and experimental models (Cowley, Gudapaty et al. 1993; Schafer, Gretz et al. 1994; Zheng, Wolfe et al. 2003; Ta, Harris et al. 2013; Karihaloo 2015). Higher inflammatory infiltrates and chemokine expression have been reported in male Han:SPRD Cy rats (an ADPKD model) compared to female rats; male rats develop more rapidly progressive disease than females (Cowley, Gudapaty et al. 1993; Cowley, Ricardo et al. 2001; Zheng, Wolfe et al. 2003). These observations suggest a role for inflammatory processes in the pathogenesis of ADPKD (Grantham 1997; Zheng, Wolfe et al. 2003). These include roles for pro-inflammatory chemoattractants (e.g.MCP-1), cytokines (e.g. TNF-aa) as well as pro-inflammatory signalling mediators (e.g. nuclear factor-kB and JAK-STAT) in ADPKD (Ta, Harris et al. 2013).

MCP-1 is a pro-inflammatory chemokine which recruits inflammatory cells including monocytes to an inflammatory site (Yadav, Saini et al. 2010; Ta, Harris et al. 2013). Upregulation of MCP-1 mRNA and protein levels have been observed in the tubular basement membrane and cystic fluid or tissue of human and animal models of ADPKD (Cowley, Ricardo et al. 2001; Zheng, Wolfe et al. 2003). In addition, patients with ADPKD excrete higher levels of urine MCP-1 when compared to non-ADPKD patients (Zheng, Wolfe et al. 2003; Kawano, Muto et al. 2015). Of interest, urine MCP-1 at 3 years was significantly lower in tolvaptan-treated patients from the TEMPO 3:4 trial compared to their baseline readings or to placebo-treated patients (Grantham, Chapman et al. 2016).

Besides MCP-1, other inflammatory factors and pathways reported to be upregulated in cystic fluid and tissue of experimental models of ADPKD include IL-1β, IL-2, IL-6 and TNFα (Gardner, Burnside et al. 1991; Li, Magenheimer et al. 2008; Menon, Rudym et al. 2011); osteopontin (Cowley, Ricardo et al. 2001); nuclear factor-kB (Qin, Taglienti et al. 2012); JAK-STAT (Bhunia, Piontek et al. 2002). In intervention experiments, anti-inflammatory drugs, namely methylprednisolone, rosiglitazone and etanercept, attenuated ADPKD progression (Gattone, Cowley et al. 1995; Li, Magenheimer et al. 2008; Dai, Liu et al. 2010). Conversely augmentation of the inflammatory process using exogenous cytokine (namely TNF-α) or inflammatory factor agonists (CXCR2 agonists) promoted renal cysts growth (Amura, Brodsky et al. 2008; Li, Magenheimer et al. 2008).

Table 1.5. Chemokine subfamilies and their receptors

Chemokine family	Receptor	Ligands
CXC	CXCR1	CXCL8, CXCL6
	CXCR2	CXCL1, CXCL2. CXCL3, CXCL5, CXCL6, CXCL7, CXCL8
	CXCR3A	CXCL9, CXCL10, CXCL11
	CXCR3B	CXCL9, CXCL10, CXCL11, CXCL4
	CXCR4	CXCL12
	CXCR5	CXCL13
	CXCR6	CXCL16
	CXCR7	CXCL11, CXCL12
CC	CCR1	CCL3, CCL3L1, CCL5, CCL7
	CCR2A/B	CCL2, CCL7, CCL8, CCL13
	CCR3	CCL3L1, CCL5, CCL7, CCL11, CCL13, CCL28
	CCR4	CCL17, CCL22
	CCR5	CCL3, CCL3L1, CCL4, CCL4L1, CCL5
	CCR6	CCL20
	CCR7	CCL19, CCL21
	CCR8	CCL1
	CCR9	CCL25
	CCR10	CCL27, CCL28
XC	CXR1	XCL1, XCL2
CX_3C	CX_3CR1	CX_3CL1

The table shows chemokine subfamilies and their corresponding receptors. Various chemokines bind many receptors and most of the receptors bind more than one chemokines (Chung and Lan 2011; Comerford and McColl 2011).

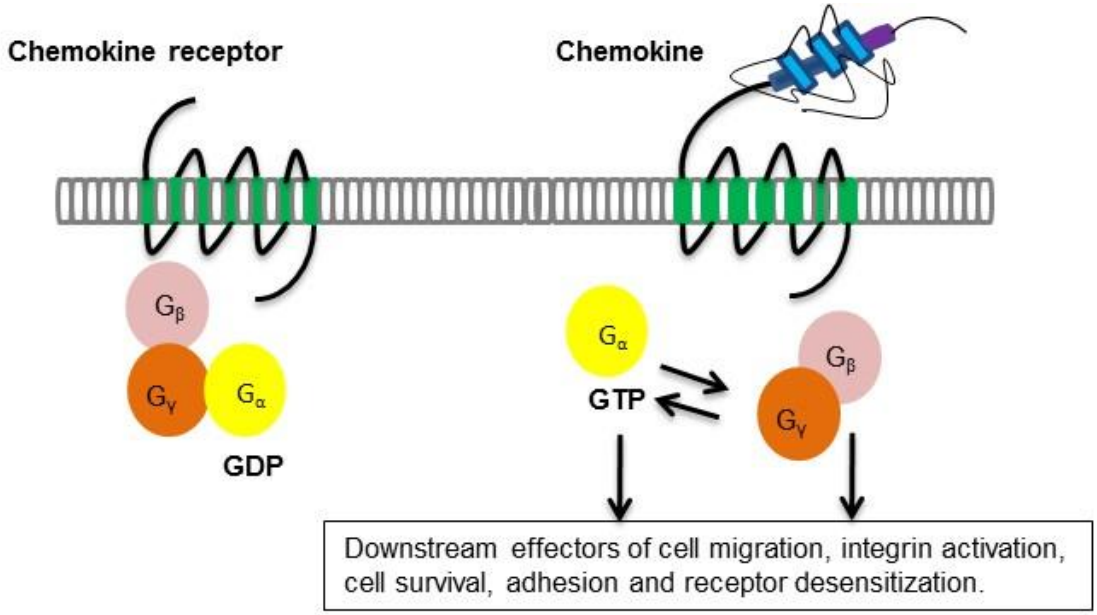

Figure 1.6. Chemokine and chemokine receptor on cell surface

The chemokine receptors are heterotrimeric G protein-coupled receptors with 7 transmembrane domains, and they express mainly on the leukocyte surfaces. Chemokines binding to their corresponding receptors lead to dissociation of the heterotrimeric G-protein complex into the Gα, Gβ and Gγ subunits. The activation of these subunits plays a significant role in stimulation of various signals that driven by chemokines. The figure was redrawn from Comerford et al. (Comerford and McColl 2011) with permission.

Genetic and epigenetic factors

The variability of ADPKD severity between patients with identical mutations of *PKD1* or *PKD2* suggests that other genetic, epigenetic and environmental factors may contribute to disease severity and progression (Magistroni, He et al. 2003). Technical advances in molecular and computational biology have allowed the identification of multiple altered gene expression pathways in ADPKD disease tissue (Husson, Manavalan et al. 2004; Song, Di Giovanni et al. 2009). A number of candidate modifier genes have been studied. For instance, expression of the CD14 gene (regulating the innate immune response) in PKD mice and in human cystic kidneys is altered (Zhou, Ouyang et al. 2010). CD14 translation, activation and expression are altered in the renal tubule epithelial cells of the cpk mouse model as well as in human ADPKD tissue. Moreover, urinary excretion of CD14 in patients with ADPKD (n = 16) correlated significantly with the rate of TKV change over 2 years (Zhou, Ouyang et al. 2010).

Contradictory results about the role of ACE polymorphisms in disease progression have been reported in the literature (Lee, Kim et al. 2000; van Dijk, Breuning et al. 2000; Schiavello, Burke et al. 2001; Persu, El-Khattabi et al. 2003). Similarly, conflicting results have been reported regarding the role of angiotensinogen gene polymorphisms in progression of ADPKD (Lee and Kim 2003; Azurmendi, Fraga et al. 2004). Other genes that have been linked to ADPKD progression include HNF4α (Menezes, Zhou et al. 2012); renin (REN) (Ramanathan, Elumalai et al. 2016); and endothelial nitric oxide synthase (NOS) (Xue, Zhou et al. 2014).

Beside genetic factors, epigenetic factors may contribute to the pathogenesis of ADPKD. Epigenetic factors can be defined as heritable changes in gene expression and chromatin remodelling which do not involve concomitant alterations in the DNA sequence (Reddy and Natarajan 2015; Hilliard and El-Dahr 2016); the epigenetic factors including DNA methylation and histone modification through phosphorylation, methylation and acetylation (Peterson and Laniel 2004; Wing, Ramezani et al. 2013; Li 2015). Perturbations in epigenetic factors have been connected to a number of pathological conditions including cancer (Feinberg, Koldobskiy et al. 2016); diabetic nephropathy (Krupa, Jenkins et al. 2010); and acute renal injury (Bomsztyk, Mar et al. 2015; Reddy and Natarajan 2015). The field of epigenetics has attracted much interest because its effects may be reversible and therefore potential targets for therapeutic intervention (Hilliard and El-Dahr 2016). In one study, reversal of histone modification in the db/db mouse model of diabetic nephropathy was achieved using losartan (Reddy, Sumanth et al. 2014).

In ADPKD, significant methylation changes at various loci including 11,999 hypermethylated and 1,228 hypomethylated fragments were found in cystic tissue from patients with ADPKD compared to malignant or healthy kidney tissues (Woo, Bae et al. 2014). This study found epigenetic silencing of genes involved in key pathways previously implicated in ADPKD disease progression such as Wnt, Notch and mTOR signalling (Woo, Bae et al. 2014). Hypermethylation of the mucin-like protocadherin gene (MUPCDH) stimulated proliferation of human cystic epithelial cells (Woo, Shin et al. 2015), moreover, excretion of DNA methylation levels of MUPCDH promotor regions in urine samples from patients with ADPKD (n = 53) was associated with the rate of disease progression (Woo, Shin et al. 2015). Beneficial effects of epigenetic factors manipulation have been reported in experimental models (Fan, Li et al. 2012; Woo, Shin et al. 2015). For instance, in a non-orthologous animal model of ADPKD, pharmacological inhibition of histone deacetylases (HDACs) has been reported to decrease cyst growth and the rate of renal function decline (Li 2011); HDACs cooperates with p53 to repress *PKD1* gene transcription (Van Bodegom, Saifudeen et al. 2006). Epigenetic regulation of other mechanisms involved with ADPKD progression (Li 2015) such as hypertension (Lee, Cho et al. 2012), interstitial inflammation (Chen, Guo et al. 2014) and interstitial fibrosis (Bechtel, McGoohan et al. 2010) have been reported.

MicroRNAs

MicroRNAs are short (19-25 nucleotides) non-coding single strand RNAs (Li, Yong et al. 2010; Edelstein 2011). The first microRNA, Line-4, was discovered in Caenorhabditis elegans (Lee, Feinbaum et al. 1993). MicroRNAs are expressed in various species, and their expression patterns are difference between the species and even during embryogenesis in each species (Lau, Lim et al. 2001; Bartel 2009). To date, more than 1000 microRNAs that regulate the human transcriptome have been identified (Bartel, 2009; Krol et al., 2010; MacFarlane and Murphy, 2010). A characteristic feature of microRNA expression is tissue specificity, making them perfect diagnostic biomarkers (Li, Yong et al. 2010).

The small molecular weight of microRNAs also protects them from endogenous RNases (Li, Yong et al. 2010). In comparison to other RNAs, microRNAs are very stable in biofluids and tissues (Jung, Schaefer et al. 2010; Aryani and Denecke 2015). The remarkable stability of microRNAs in urine samples have positioned microRNAs profiling as promising biomarkers for clinical applications. However, despite the role of

transcriptomic analysis in understanding the pathophysiological events in different diseases, transcriptomic studies alone cannot provide information regarding post-translation modifications that could alter protein function (Cao, Lu et al. 2012). The kidney expression of microRNAs has been found to differ from other organs and even differs between nephron segments (Li, Yong et al. 2010). Using microarrays, Tian and colleagues analysed microRNA expression of the medulla and cortex of a rat kidney (Tian, Greene et al. 2008). Out of 377 analysed microRNAs, 6 were identified as enriched in the cortex whereas 11 were enriched in the medulla. The findings in this study demonstrated a probable role for microRNA expression in nephron specification (Li, Yong et al. 2010).

MicroRNA biogenesis begins in the nucleus through the transcription of microRNA genes by RNA polymerase-II to produce primary microRNAs, which are then processed by Drosha, a ribonuclease enzyme, into precursor microRNAs (approximately 70 nucleotides) (Chandrasekaran, Karolina et al. 2012) (**Figure 1.7**). Pre-microRNAs are exported to the cytoplasm through exportin 5 (Chandrasekaran, Karolina et al. 2012). In the cytoplasm, pre-microRNAs are processed by another ribonuclease enzyme, Dicer, to a mature microRNA. Mature microRNAs are small, double-stranded RNA duplex molecules, microRNA guide strand and it's complementary that is usually subject to degradation (Iorio and Croce 2012). Detection of both strands at significant levels has been reported (Hu, Yan et al. 2009; Woo and Park 2013). The microRNA guide strand incorporates into an RNA-induced silencing complex (RISC) and its 5' end binds to target messenger RNAs via complementary sequences on their 3' untranslated regions (Chandrasekaran, Karolina et al. 2012). The formation of microRNA-mRNA bond depends on distinctive conservative complementary sequence within the microRNA seed (Lewis, Burge et al. 2005; Grimson, Farh et al. 2007). The fate of this binding is based on the level of complementation between a microRNA and its target mRNA (Pillai 2005). A complete destruction of a target mRNA will occur if there is a perfect complementation with the target microRNAs (Hutvagner and Zamore 2002; Pillai 2005). Conversely, the presence of imperfect complementation between a microRNA and its target mRNA will inhibit protein translation (Bartel 2004; Pillai 2005). A positive regulation of mRNA, via increase expression or stimulate translation, by its target microRNAs has been described (Orom, Nielsen et al. 2008; Place, Li et al. 2008). A single microRNA can target thousands of genes, approximately 60% of human protein encoding genes, whereas, each mRNA may possess numerous binding sites for microRNAs (Lewis, Burge et al. 2005; Friedman, Farh et al. 2009; Hajarnis, Lakhia et al. 2015).

Many studies have investigated the importance of urinary microRNAs as biological biomarkers for various systemic diseases such as in cancer, cardiac and liver disease (Abdalla and Haj-Ahmad 2012; Snowdon, Boag et al. 2012; Zhou, Mao et al. 2013). Urinary microRNAs have also been investigated as potential biomarkers in a number of renal diseases such as lupus nephritis, IgA nephropathy, acute allograft nephropathy and chronic allograft nephropathy (**Table 1.6**). Among these microRNAs, 27 urinary microRNAs were described as biomarkers for different stages of diabetes nephropathy (DN) (Argyropoulos, Wang et al. 2013); whereas urinary miR-210, miR-10b and miR-10a were found deregulated in acute renal allograft rejection (Lorenzen, Volkmann et al. 2011). Urinary miR-146a and miR-155 levels were increased in patients with lupus nephritis compared with healthy participants (Wang, Tam et al. 2012); urinary miR-216a levels correlated with the rate of kidney function decline in patients with hypertensive nephrosclerosis and diabetic glomerulosclerosis (Szeto, Ching-Ha et al. 2012).

Previous studies in animal or cell culture models of ADPKD have linked a number of microRNAs to ADPKD pathogenesis (**Table 1.7**). MicroRNAs were reported to be contributed to the pathogenesis of cystogenesis via regulation the expression of PKD genes and also through the regulation of renal tubular development (Noureddine, Hajarnis et al. 2013). MiR-17 represses a post-transcriptionally expression of *PKD2* in cystic tissue and also antagonised the effects of Bicaudal C on *PKD2* (Sun, Li et al. 2010; Tran, Zakin et al. 2010). MiR-15a modulates the expression of Cdc25A, a key regulator of cell cycle, in liver tissue of PCK rate mode of ARPKD; this effect has been linked to the pathogenesis of hepatic cystogenesis (Lee, Masyuk et al. 2008). The role of microRNAs in the development of renal tubules and maintenance of their integrity has been reported (Nagalakshmi, Ren et al. 2011; Noureddine, Hajarnis et al. 2013). Additionally, a miR 200 family, including miR-200b, 200c and 429, regulates renal tubular maturation via represses a post-transcriptionally expression of *PKD1* (Patel, Hajarnis et al. 2012). Over expression of oncogene microRNA cluster (miR-17~92) promotes renal cyst growth in Kif3a-KO mice model of ADPKD via repression of post-transcriptionally expression of *PKD1*, *PKD2* and hepatocyte nuclear factor-1β (Patel, Williams et al. 2013). On the other hand, knockout of miR-17~92 in the same ADPKD retards renal cyst growth and improve kidney function (Patel, Williams et al. 2013).

A microRNA based therapeutic approach has been suggested using microRNA antagonists or mimics (Noureddine, Hajarnis et al. 2013). These therapeutic techniques have been applied in treatment of HCV infection (Janssen, Reesink et al. 2013); hypercholesterolemia (Irani, Pan et al. 2016); prostate cancer (Takeshita, Patrawala et al. 2010); and lung cancer (Esquela-Kerscher, Trang et al. 2008).

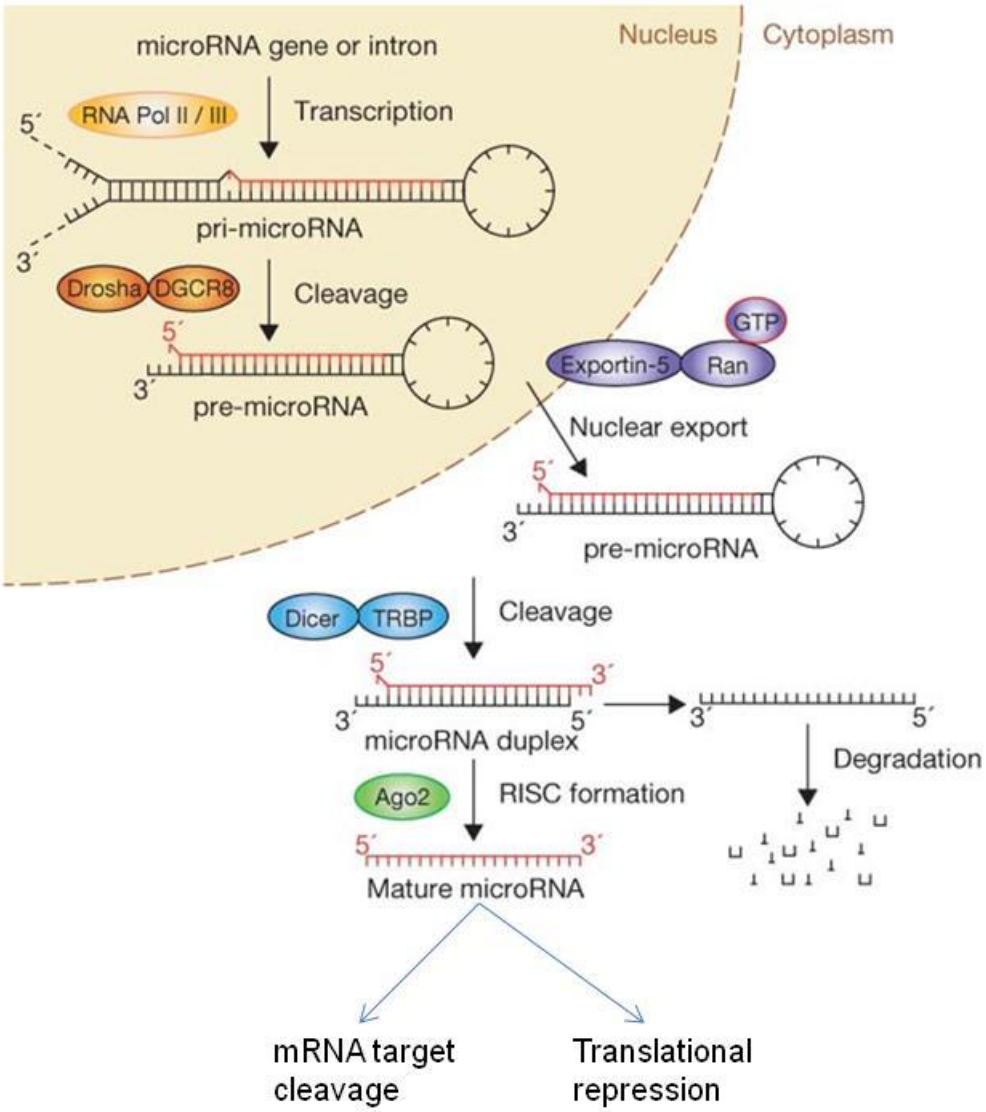

Figure 1.7. MicroRNAs biogenesis

The figure was modified from Winter et al. (Winter, Jung et al. 2009) with permission.

Table 1.6. Urinary microRNAs in renal disease

MicroRNAs	Expression	Experimental model	Comment	Reference
MiR-200a, 200b and 429	Down	Patients with IgA nephropathy (n = 43)	MicroRNAs are negatively correlated with the disease severity and progression.	(Wang, Kwan et al. 2010)
MiR-10a, 10b and 210	Down; MiR-10b and 210. Up; miR-10a	Patients with acute renal allograft rejection (n = 62)	MiR-210 levels predict long-term renal function.	(Lorenzen, Volkmann et al. 2011)
MiR-146a and 155	Up	Patients with lupus nephritis.	A potential biomarker for disease activity	(Wang, Tam et al. 2012)
MiR-10a and 30d	Up	Serum, urine, and kidney tissue of C57BL/6J mice	urinary levels of miR-10a and 30d levels were more sensitive for the detection of AKI when compared to serum levels of same microRNAs.	(Wang, Zhou et al. 2012)
Serum miR-30a-5p, 151-3p, 150, 191 and 19b Urine miR-30a-5p	Up	Children with NS (n = 159), healthy controls (n = 109) and children with other kidney diseases (n = 44)	Prognostic biomarkers for idiopathic NS	(Luo, Wang et al. 2013)
MiR-146a	Up	Patients with SLE (n = 38)	Discriminates the patients with active LN when compared to the patients with inactive disease.	(Perez-Hernandez, Forner et al. 2015)

Abbreviations are: SLE; Systemic Lupus Erythematosus, LN; Lupus Nephritis, NS; Nephrotic Syndrome.

Table 1.7. MicroRNAs in ADPKD

MicroRNAs	Expression	Experimental PKD model	Relation to ADPKD	Target gene and pathway	Reference
MiR-21, 302c, 31,217, 34b, 126, 7, 128b, 302b, 136, 99a, 448, 380-3p, 20, 96, 372, 76, 379, 203, 147, 196a, 335, 216, 128a, 30a-3p, 148a, 181b, 346 and 377	Mostly down	Han:SPRD rats	Regulatory roles in cell proliferation and cysts formation	11 genes 5 pathways	(Pandey, Brors et al. 2008)
MiR-15a	Down	PCK rats	Regulatory roles in cell proliferation and cysts growth	Cdc25A	(Lee, Masyuk et al. 2008)
MiR-17	/	HEK 293T cell line	Stimulate cell proliferation	3′UTR of *PKD2*	(Sun, Li et al. 2010)
MiR-17	/	Bicc1−/− mice HEK293T cells	Bicc1 antagonised the effects of miR-17 on *PKD2*	Bicc1	(Tran, Zakin et al. 2010)
MiR-10a, 30a-5p, 126-5p, 182, 200a, 204, 429 and 488	All up-regulated except miR-488 and 204	*PKD1*$^{-}$/$^{-}$mice	/	16 genes	(Pandey, Qin et al. 2011)
MiR-200b, 200c and 429	Down	Dicer mutant mice	Renal tubules maturation	3′UTR of *PKD1*	(Patel, Hajarnis et al. 2012)
MiR-17, 18a, 19a and 20a	Up	Kif3a-KO mice	Promotes proliferation and cyst growth	/	(Patel, Williams et al. 2013)
rno-miR-21, 31, 34a, 199a-5p, 132, 146b, 214 and 503	Up	PKD/Mhm rats	Associated with PKD regulating pathways	17 significantly enriched pathways	(Dweep, Sticht et al. 2013)
MiR-1, 133, 199 and 223	Down; miR-1 and 133 Up; miR-199and 223	Urine specimens of ADPKD patient (n = 20) and CKD patients (n = 20)	MicroRNAs profiling at baseline	/	(Ben-Dov, Tan et al. 2014)
MiR-21	Up	Ksp/Cre;Kif3aF/F mice	Promotes disease progression	cAMP pathway	(Lakhia, Hajarnis et al. 2016)

Abbreviations are: 3′UTR; 3′ untranslated regions, Bicc1; Bicaudal C, Cdc25A; cell-cycle regulator cell division cycle 25A.

Metabolomics

Many exogenous and endogenous chemical substances are included in metabolic profiles. These include peptides, nucleic acids, amino acids and drug metabolites (Edelstein 2011). Metabolites typically have very low molecular weights (less than 1 kDa) (Edelstein 2011). Nevertheless, metabolic changes can occur within a short time (less than an hour), while changes in protein and gene expression profiles usually take hours or days (Edelstein 2011). Different regions in the kidney face unique metabolic challenges because of differences in oxygen tension and tonicity. Therefore, metabolomics could potentially differentiate functional changes arising from different parts of the kidney; for instance, the expression of Krebs' cycle enzymes is relatively high in renal cortex whereas the concentrations of anaerobic glycolysis enzymes are relatively low (Edelstein 2011). However, practical metabolomics analysis procedures are technically difficult and costly. In addition, the analytical methods are considered immature in comparison with gene arrays (Edelstein 2011). Metabolic profiles can be affected by endogenous and exogenous factors; therefore, possible confounders should be considered in the interpretation of results, for instance due to changes in diet and exercise (Edelstein 2011).

Several studies have concluded that human and animal models of ADPKD share common cystogenesis mechanisms (Weiss and Kim 2012). Based on this assumption, a study using gas chromatography-mass spectrometry (GSMS) to identify urinary metabolites was able to discriminate cystic (juvenile jck mice) from wild-type mice (Taylor, Ganti et al. 2010). Despite the normal renal function of cystic mice at day 26, several metabolic pathways were found to be altered and this distinguished them from wild-type mice. Urinary adenosine and allantoin were significantly increased in cystic compared to non-cystic mice. These results have not yet been confirmed in human PKD.

The EGF/ErbB receptor family

As mentioned early in the section 1.5.1, the epidermal growth factor receptor family (EGFR) plays a significant role in the pathogenies of ADPKD. EGFR belongs to the tyrosine kinase receptor superfamily (Schlessinger 2002). It consists of four members i.e. ErbB1, ErbB2, ErbB3 and ErbB4, with a common structure that comprises an extracellular ligand binding domain, a single membrane domain and a cytoplasmic tyrosine kinase domain (Staruschenko, Palygin et al. 2013). A number of different ligands can interact with EGFR (except ErbB2) including transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF) and neuregulin 1-4 (NRG1-4). Binding of a ligand to the EGFR stimulates its phosphorylation on specific tyrosine residues within the cytoplasmic tail that leads to the activation of several signalling pathways including JAK/STAT, MAPK and PI3K (**Figure 1.8**) which in turn regulate cell division, differentiation, migration and apoptosis (Riese and Stern 1998; Hynes and Lane 2005; Veikkolainen, Vaparanta et al. 2011; Tang, Liu et al. 2013).

ErbB4 also undergoes alternative splicing to generate several isoforms that differ in the intracellular domain (CYT-1 and CYT-2) and extracellular domain (JM-a and JM-b) (Sundvall, Peri et al. 2007). JM-a is prone to proteolytic cleavage as it contains a cleavage site for a proteinase missing in JM-b (Zeng, Miyazawa et al. 2014). CYT-1 has a 16 aa sequence that serves to mediate PI3K signalling (Elenius, Choi et al. 1999). CYT-1 is more prone to degradation by ubiquitination. CYT-2 has significantly higher tyrosine kinase activity and undergoes nuclear translocation in comparison to CYT-1 (Vecchi and Carpenter 1997; Sundvall, Peri et al. 2007; Zeng, Xu et al. 2009). ErbB4 CYT-1 is predominantly expressed in the heart and mammary tissue whereas ErbB4 CYT-2 is mostly expressed in the neural and renal tissues (Elenius, Choi et al. 1999).

The ErbB/EGF family plays crucial roles during embryonic development; loss of a member of ErbB family leads to lethality of embryonic mice with various organ defects depending on the mutated receptor (Olayioye, Neve et al. 2000; Cho and Leahy 2002). The ErbB family is widely expressed in epithelial, mesenchymal as well as neuronal tissues and is highly expressed in various compartments of the kidneys including glomerular mesangial cells, renal tubules and interstitial cells (Plowman, Culouscou et al. 1993; Hynes and Lane 2005; Zeng, Singh et al. 2009; Tang, Liu et al. 2013). Insufficient ErbB signalling has been linked to various neurodegenerative diseases including Alzheimer disease, schizophrenia and neurones demyelination in multiple sclerosis or leprosy (Bublil and Yarden 2007). On the other hand, excessive ErbB signalling has been linked to hypertension, acute and chronic kidney impairment, and various solid tumours including breast and lung cancers (Bublil and Yarden 2007;

Staruschenko, Palygin et al. 2013; Tang, Liu et al. 2013; Tang, Liu et al. 2013; Zhuang and Liu 2014).

The role of EGFR/ErbB in the pathogenesis of ADPKD has been extensively studied in ADPKD (**Table 1.8**). It can regulate ENaC-mediated tubular sodium transport (Zheleznova, Wilson et al. 2011). In addition, EGF has been reported to stimulate cystic epithelial cells proliferation in both ADPKD and ARPKD (Wilson, Du et al. 1993; MacRae Dell, Nemo et al. 2004; Zheleznova, Wilson et al. 2011). Changes in planar cell polarity are associated with tubular dilatation and ErbB4 has been shown to modulate tubular cell polarity (Fischer, Legue et al. 2006; Veikkolainen, Naillat et al. 2012). EGFR was found to be mislocalised to the apical surface of cystic tubules from its normal basolateral location (Du and Wilson 1995). In some models, blockade of EGFR improved renal function and retarded cyst growth (Richards, Sweeney et al. 1998; Sweeney, Chen et al. 2000; Sweeney, Hamahira et al. 2003; Zeng, Miyazawa et al. 2014). These results support a pathogenic role for significant increases in EGFR ligands found in PKD cystic tissue. These include NRG-1, EGF, HB-EGF and TGF-a (Lee, Chan et al. 1998; MacRae Dell, Nemo et al. 2004; Tang, Liu et al. 2013).

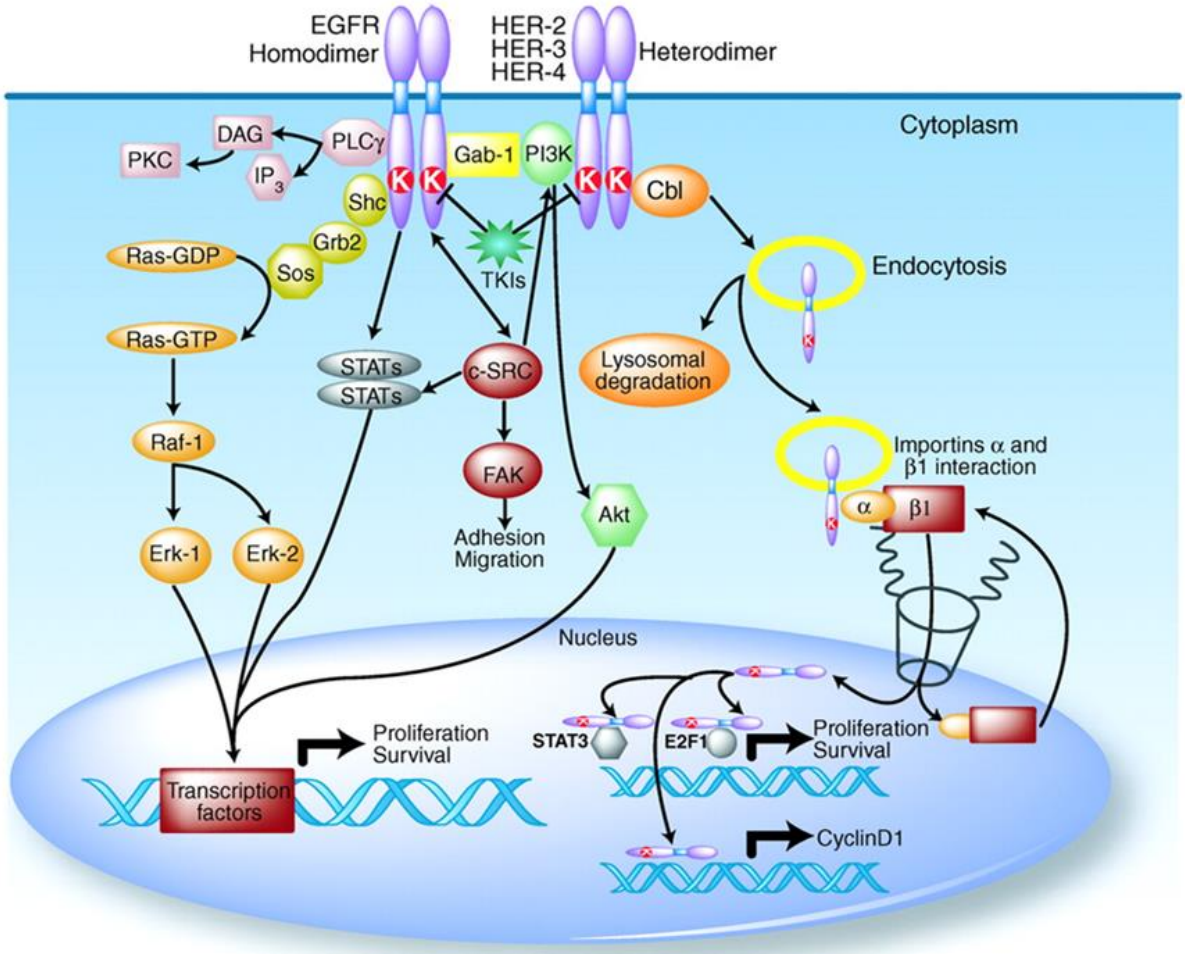

Figure 1.8. EGFR signalling pathways

Ligands binding to the EGFR family leads to homo and heterodimer formation and subsequently phosphorylation and activations of tyrosine kinase domain on the cytoplasmic domain, and this lead to cascade activation of various signalling including STAT transcription factor, Ras/Raf/MAPK pathway and PI3K. The figure was reproduced from Scaltriti et al. (Scaltriti and Baselga 2006) with permission.

Table 1.8. EGFR/ErbB role in the pathogenesis of ADPKD

PKD model	Relation to ADPKD	Reference
Bpk mice model of ADPKD	Over-expression of EGFR, EGFR, HB-EGF and ErbB4 in cystic tissue was reported.	(Nemo, Murcia et al. 2005)
Cystic transgenic mice (Hoxb7/GFP-bpk+/+)	Mislocalization of EGFR to the apical surface of renal epithelial cells may contribute to ADPKD pathogenesis.	(Veizis and Cotton 2005)
Cpk mice model of ADPKD	Low expression level of ErbB4 may contribute to the pathogenesis of cystogenesis.	(Zeng, Miyazawa et al. 2014)
Mice with conditional knockout of ErbB4 (Pax8-Cre)	Over expressions of ErbB4 isoforms enhance proliferation, alter polarization and stimulate formation of tubular cysts.	(Veikkolainen, Naillat et al. 2012)
MDCK II cell lines	ErbB4 isoforms selectively mediate the stimulation of tubulogenesis by growth factors in renal epithelial cells of Madin-Darby canine.	(Zeng, Zhang et al. 2007)
Human ADPKD kidney	EGFR protein is highly expressed in cystic epithelial cells.	(Du and Wilson 1995)
Human ADPKD kidney	EGF are highly mitogenic to the cystic epithelial cells.	(Wilson, Du et al. 1993)
PKD1 null heterozygous mice	Inhibition of ErbB2 restore normal renal function and structure in a mice model of the disease.	(Wilson, Amsler et al. 2006)
Genotyping of 46 patients with ADPKD	EGFR polymorphism is associated with the pathogenesis of ADPKD.	(Magistroni, Manfredini et al. 2003)

The table summarises previous EGFR/ErbB studies in ADPKD.

Angiogenesis

Angiogenesis refers to the process of new capillary growth which originates from pre-existing vessels (Persson and Buschmann 2011). Under physiological conditions, this process occurs mainly during embryogenesis, reproduction and tissue repair (Reynolds, Killilea et al. 1992; Breier, Damert et al. 1997). In normal cells, angiogenesis is tightly controlled through maintaining the balance between angiogenesis stimulators and inhibitors (Chung, Lee et al. 2010). **Table 1.9** summarises the well-characterized angiogenic factors in the literature. This process starts by the degradation of the vascular basement membrane by matrix metalloproteinases secreted by endothelial cells. Disruption of the basement membrane allows endothelial cells to migrate and proliferate though the action of pro-angiogenic factors (**Figure 1.9**) (Bussolino, Mantovani et al. 1997; Bello-Reuss, Holubec et al. 2001).

The key regulator of angiogenesis is VEGF, also called vascular permeability factor (Senger, Galli et al. 1983; Ferrara 2001). The VEGF family consists of 7 members i.e. VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor, and all members have a common VEGF homology domain (Hoeben, Landuyt et al. 2004). Each member of this family seems to have a specific function: VEGF-A induces angiogenesis, VEGF-B plays a redundant role for VEGF-A, VEGF-C and VEGF-D act predominantly as lymphangiogenic factors, VEGF-E involves in the process of pathological angiogenesis in virus-infected lesions and PlGF mediates both angiogenesis and arteriogenesis (Hoeben, Landuyt et al. 2004; Testa, Pannitteri et al. 2008; Liu, Xu et al. 2015).

Abnormal angiogenesis is results from imbalance of pro- and anti-angiogenic factors and occurs in many pathological conditions including rheumatoid arthritis, psoriasis, proliferative retinopathy, and in the development and progression of cancer (Folkman 1995; Maeshima and Makino 2010). In cancer tissues, the density of microvessels is an independent predictor of poor outcome (Weidner, Semple et al. 1991; Macchiarini, Fontanini et al. 1992; Wakui, Furusato et al. 1992; Poon, Ng et al. 2002). In addition, over-expression of some angiogenic factors in cancer tissue such as VEGF (Seo, Baba et al. 2000); PDGF (Matsumura, Chiba et al. 1998); and FGF (Yamanaka, Friess et al. 1993) correlated with tumour stages and progression rates.

Angiogenesis related proteins have been shown to play a role in kidney development and specifically in maintaining the structure of capillary basement membranes (Kitamoto, Tokunaga et al. 1997; Del Porto, Mariotti et al. 1999; Tufro, Norwood et al. 1999; Kim and Goligorsky 2003; Maeshima and Makino 2010). In CKD, defective

angiogenesis may lead to a reduction in renal blood flow preceding the onset of tubule-interstitial fibrosis (Futrakul, Yenrudi et al. 2000; Futrakul, Butthep et al. 2008; Tanaka and Nangaku 2013). However, an increase in the number of glomerular capillaries as well as VEGF-A and its receptor has been found in the early stages of diabetic nephropathy (Nyengaard and Rasch 1993; Cooper, Vranes et al. 1999). A number of studies have described the prognostic value of different pro-angiogenic factors in a variety of chronic kidney diseases (**Table 1.10**).

In ADPKD, kidney volume may expand by over 10 fold (Wei, Popov et al. 2006) due to progressive cyst growth. This can disturb the supply of oxygen and nutrients to the growing cysts and surrounding structure. Typically, pericystic hypoxia leads to upregulation of hypoxia-inducible transcription factors (HIF-1α and HIF-1β) which are potent stimulators for several angiogenic factors (Haase 2006; Bernhardt, Wiesener et al. 2007). Chronic expression of HIF in cystic epithelial cells and pericystic interstitial cells has been reported (Bernhardt, Wiesener et al. 2007). Two independent morphological studies have reported a disorganized pattern of tortuous vessels around the renal cysts indicating abnormal angiogenesis (Bello-Reuss, Holubec et al. 2001; Wei, Popov et al. 2006). High levels of angiogenic factors have been reported in cystic fluid, serum and urine of human and experimental models of ADPKD (Nichols, Gidey et al. 2004; Reed, Masoumi et al. 2011; Raina, Lou et al. 2016). The altered expression of pro-angiogenic factors in the early stages of ADPKD has led to their study as significant roles in disease progression (Bernhardt, Wiesener et al. 2007; Reed, Masoumi et al. 2011). Anti-angiogenic therapy (i.e. blocking vascular endothelial growth factor A) in a non-orthologous rat model of ADPKD has however given conflicting results (Tao, Kim et al. 2007; Raina, Honer et al. 2011; Huang, Woolf et al. 2016).

Table 1.9. Inducers and inhibitors of angiogenesis

Pro-angiogenic factors	Anti-angiogenic factors
Angiogenin	Angiopoietin-2
Angiopoietin-1	Anti-thrombin III fragment
Cyclooxygenase-2	Endostatin
Epidermal growth factor	Human macrophage metalloelastase
Granulocyte colony-stimulating factor (G-CSF)	Interferon-α/β
Fibroblast growth factors	Interleukin-12
Granulocyte–macrophage colony-stimulating factor (GM-CSF)	Osteopontin fragment
Hepatocyte growth factor	Platelet factor 4 fragment
Interleukin-6/8	Plasminogen activator inhibitors (PAI-1and -2)
Interleukin 8	Prolactin
Monocyte chemoattractant protein 1 (MCP-1)	Thrombospondin
Nitric oxide	Thrombospondin-1, 2
Placental growth factor	Tissue inhibitor of metalloproteinase-1/2
Platelet-derived endothelial cell growth factor (PDGF)	Vascular endothelial growth inhibitor
Prostaglandin E1, E2	
Tissue factor	
Transforming growth factor-α/β	
Tumour necrosis factor-α	
Vascular endothelial growth factor (VEGF)	

The table summarises major angiogenic stimulator and inhibitor factors (Augustin 1998; Pang and Poon 2006).

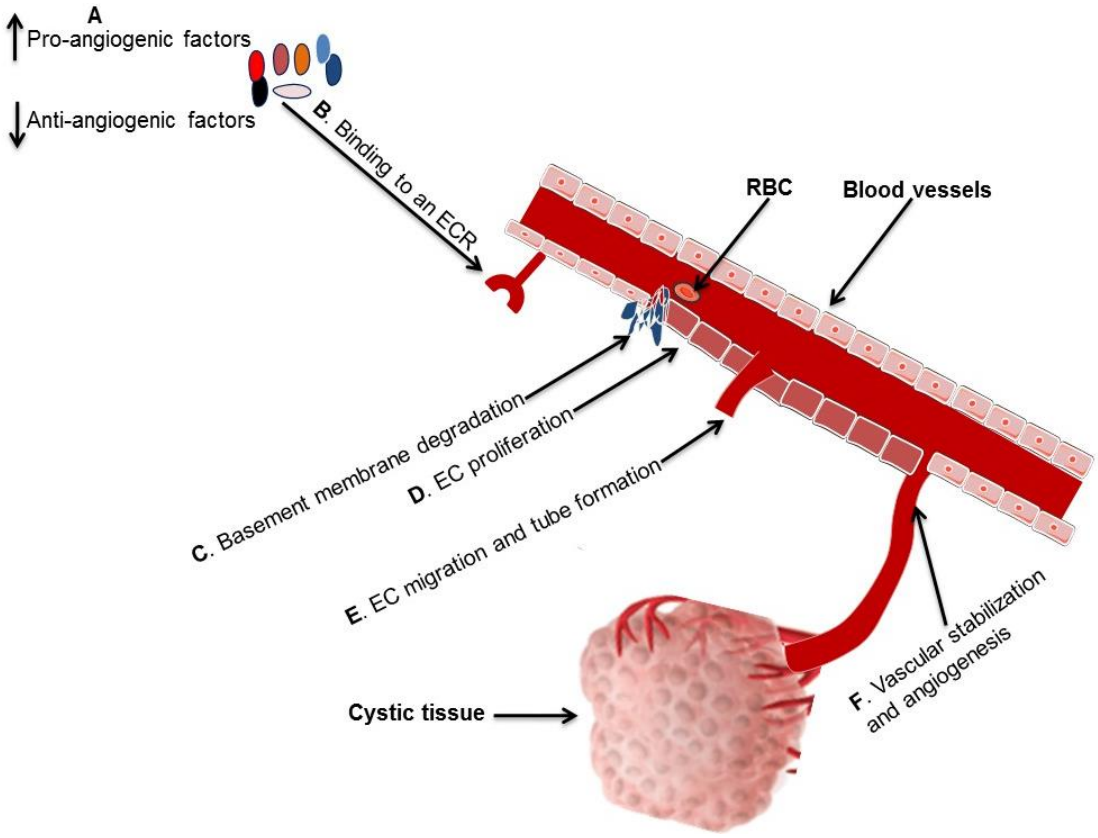

Figure 1.9. The process of angiogenesis

The figure summarizes the angiogenesis cascade events. Abbreviations are: EC; endothelial cell, ECR; endothelial cell receptor, RBC; Red blood cell.

Table 1.10. Angiogenic factors with prognostic values in renal disease

Renal disease	Angiogenic factor	Reference
CKD	Urinary epidermal growth factor	(Tsau and Chen 1999)
IgA nephropathy	Urinary transforming growth factor-beta	(Goumenos, Tsakas et al. 2002)
Immune-mediated glomerulonephritis	Tissue angiopoietin	(Yuan, Tipping et al. 2002)
DM	Urinary TNF-α	(Kalantarinia, Awad et al. 2003)
Acute kidney injury	Plasma fibroblast growth factor 23	(Christov, Waikar et al. 2013)
ADPKD	Urinary MCP-1	(Kirby, Stepanek et al. 2014)
Chronic glomerulonephritis	Urinary VEGF, angiopoietin 2 and thrombospondin 1	(Shvetsov, Zheng et al. 2015)
Chronic glomerulonephritis	Urinary MMP, TIMP and PAI-1	(Li, Bobkova et al. 2009)
ADPKD	Urinary VEGF	(Martins, Souza et al. 2016)

Abbreviations are: CKD; chronic kidney disease, DM; diabetic mellitus, TNF-α; Tumour necrosis factor-α, MMP; Matrix metalloproteinases, VEGF; Vascular endothelial growth factor, TIMP; tissue inhibitors of metalloproteinases, PVI-1; Plasminogen activator inhibitor-1.

Chapter 2 Materials and Methods

2.1 The cohort study

Ethical approval for this study was obtained from the National Research Ethics Service Committee Yorkshire and The Humber Bradford (REC12/YH/0297). ADPKD patients were recruited from the ADPKD clinic at the Sheffield Kidney Institute, NHS trust. All participants gave their signed informed consent at the time of recruitment. The approval permits the urine and blood collection from patients with renal diseases as well as healthy volunteers. Prior to their consent, the participants received a detailed explanation of the project's purpose.

Detailed clinical information of the patients was obtained from PROTON (computerized patients record system) at the Sheffield Kidney Institute, Sheffield Teaching Hospitals NHS Foundation Trust. This included age, gender, clinical examination, systemic reviews, drug history, detailed family history, biochemical investigation and radiological images. The diagnosis of ADPKD was made according to the unified ultrasonography criteria (**Table 1.2**) (Pei, Obaji et al. 2009). The inclusion and exclusion criteria specified for this study were pre-determined at the start and summarized in the **Table 2.1**.

Healthy volunteers were recruited from university staff at the Royal Hallamshire Hospital, NHS trust. The volunteers were recruited as normal controls if they had no history of known renal disease, any other systemic diseases and were not taking nephrotoxic drugs. Healthy volunteers were matched with the patient group for age, gender and ethnicity. Baseline serum and urine biochemical investigations were also measured. Notably, a number of healthy volunteers were excluded from this project because they were found to have undiagnosed concomitant disease, namely DM or renal impairment.

In this project, estimated GFR was measured using the CKD-EPI formula (Levey, Stevens et al. 2009). It has been reported that the CKD-EPI formula is more accurate than the MDRD formula (Modification of Diet in Renal Disease Study equation) especially in patients with higher GFR (Levey, Stevens et al. 2009; Madero and Sarnak 2011). The CKD-EPI equation is based on 4 variables similar to the MDRD equation, however CKD-EPI equation uses a 2-slope spline to model the relationship between eGFR and serum creatinine, and a different relationship for age, sex and race (Levey, Stevens et al. 2009). The CKD-EPI equation produces higher eGFR and therefore lower CKD estimates, particularly among patient groups (18-59 year) with MDRD eGFR of stage 3 (Carter, Stevens et al. 2011; Rosa-Diez, Varela et al. 2011). However, among

the elderly patients (>70 years) there is very little difference between the 2 equations (Carter, Stevens et al. 2011).

Table 2.1. The inclusion and exclusion criteria specified for this project

Inclusion criteria	Exclusion criteria
Caucasian race	Patients with diabetes or other coincident renal disease
Age older than 18 years	The regular use of nephrotoxic e.g. NSAIDs
A clear diagnosis of ADPKD according to ultrasound criteria	End-stage renal failure treated by renal replacement therapy, or evidence of acute kidney injury

2.2 Sample collection

The time of collection, processing and storage conditions of urine were consistent among all collected samples. Spot urine samples were collected into containers supplemented with protease inhibitors (PI), (Sigma, cat no; S8820, with a broad specificity to inhibit cysteine, serine and metalloproteases), 4.2 ml of 10 x PI for every 50 ml of urine (Zhou, Yuen et al. 2006). Each sample was then centrifuged at 1,000 x g for 10 minutes at 4^{o}C, any cell pellet discarded and the cell-free supernatant were stored at -80^{o}C until further analysis (**Figure 2.1**). Whole blood samples were collected directly into EDTA tubes to obtain plasma samples. These samples were kept on ice until processed. The serum samples were spun for 10 minutes at 5,000 x g, aliquoted and stored at -80^{o}C. As the main purpose of this project was to identify a clinical diagnostic marker, timing of voiding is a crucial aspect of clinical diagnosis, previous a study found minor consequences for timing of sample collection on microRNAs profile (Ben-Dov, Whalen et al. 2016).

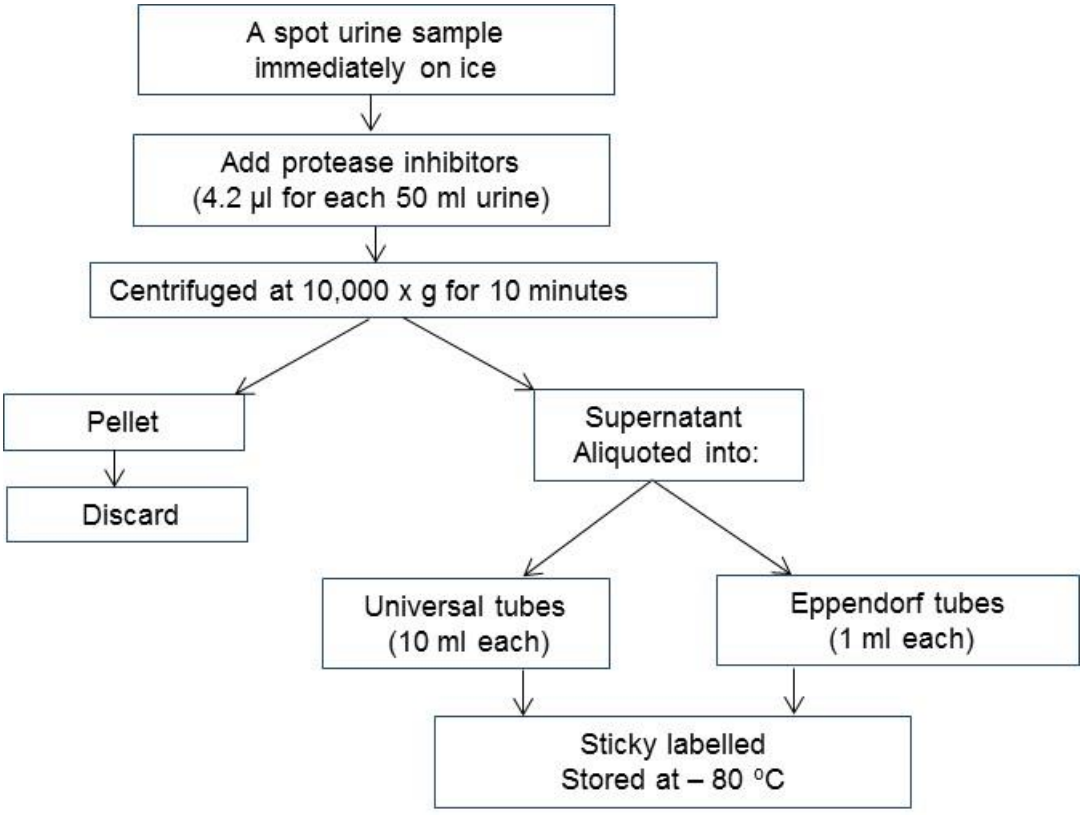

Figure 2.1. Flow chart of a spot urine sample processing

The figure shows outflow of urine samples collection and further processing.

2.3 Exosomes isolation

The most common method of exosome isolation is differential speed ultra-centrifugation (Raposo, Nijman et al. 1996; Thery, Amigorena et al. 2006). Various methods have been successfully used to isolate exosomes including ultrafiltration, nanomaterial exosome isolation using ExoQuick exosome precipitation solution, immuno-magnetic extraction using antibody coated magnetic beads raised against exosomal membrane proteins and differential centrifugation followed by sucrose cushion (Clayton, Court et al. 2001; Zhu, Qu et al. 2014; Greening, Xu et al. 2015; Lobb, Becker et al. 2015). The flow chart of exosomes isolation in this study is depicted in **Figure 2.2**.

An initial volume of 10 ml of a spot urine sample was centrifuged at 17,000 x g for 15 minutes at 24°C in an ultracentrifuge. The resulting supernatant was stored at room temperature; while the pellet was re-suspended in 200 µl isolation solution and 50 µl of DDT (**Table 2.2**). The re-suspended pellet was then incubated at room temperature for 5 minutes followed by a brief vortexing step and a second centrifugation step at 17,000 x g for 15 minutes. The resulting cell-free supernatant was mixed with the previously collected supernatant from the first step and the total solution centrifuged at 170,000 x g for 2.5 hours at 24°C (Beckman Coulter rotor 70.1Ti). The resulting pellet was then re-suspended in 50 µl lysis buffer (50 mM Tris pH 7.4, 150 mM sodium chloride, 1% Triton X-100, 1% sodium-deoxycholic acid and protease inhibitors) on ice for 1 hour (Cheng, Sun et al. 2014).

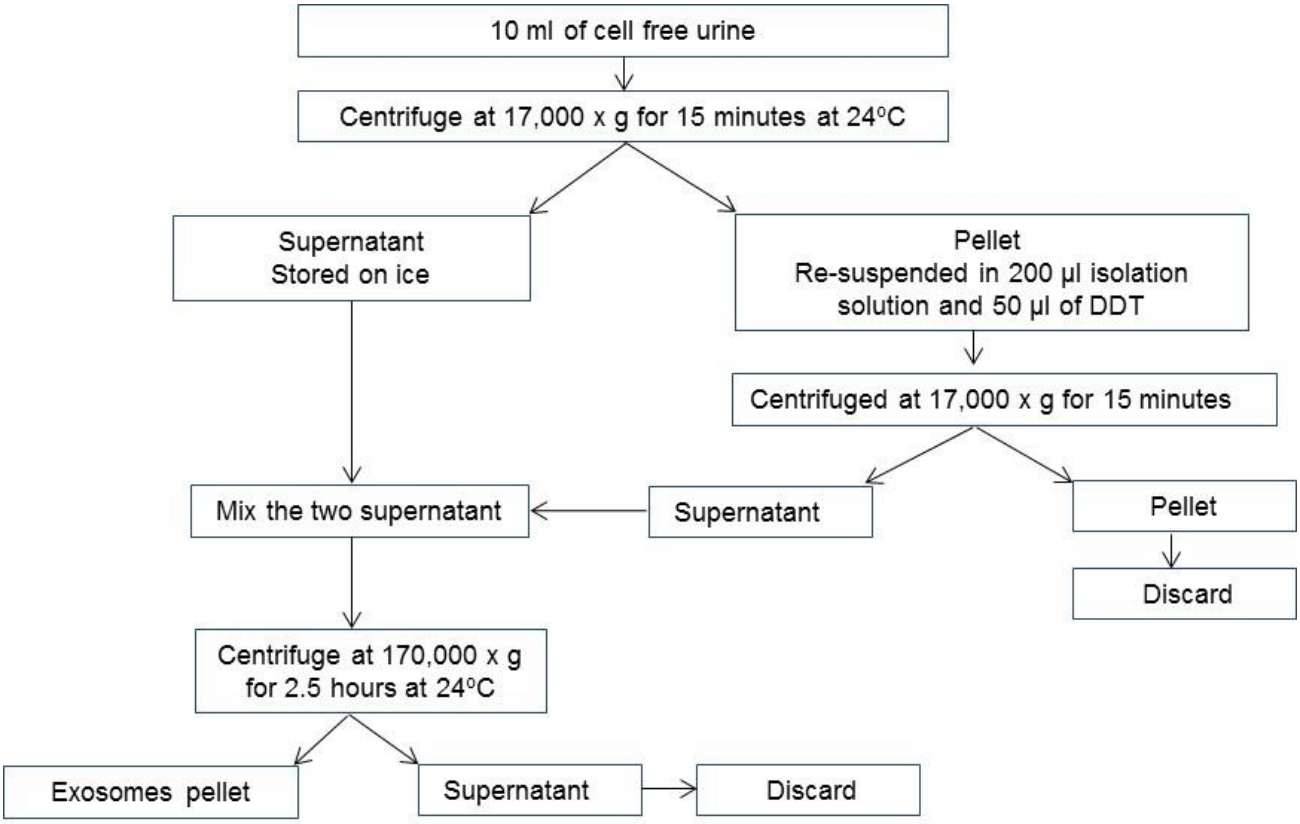

Figure 2.2. Flow chart of urine exosomes isolation

Differential ultracentrifugation was used to isolate exosomes from a spot urine sample. The graph shows the speed and duration of each centrifugation. The first pellet was discarded as it contains the large cells and debris, whereas, the final pellet was kept as it contains the small vesicles including exosomes.

Table 2.2. Exosome isolation solutions

Solution	Composition
Isolation solution	250 mM sucrose (25 ml of 0.5 M stock) + 10 Mm triethanolamine (0.5 ml of 1 M stock)+ double distilled water was added to 45 ml, pH was adjusted to 7.6 + DD water was added to make it 50 ml
DDT	Dichlorodiphenyltrichloroethane 500 mg/ml

2.4 Electron microscopy

In order to verify the purification of intact urinary exosomes, scanning electron microscopy was performed by Dr Bart Wagner, EM unit, Royal Hallamshire Hospital. Urine exosomes isolated as described in section 2.2, were re-suspended in 50 µl PBS and a small drop of this mixture was deposited on 200-mesh nickel grids. Negative staining of the mesh was performed using heavy metal salt, 0.5% Uranium. After drying, the nickel grids were visualized using a Philips electron microscope 400 operated at 80 KV.

2.5 Protein assay

After extraction, protein concentration was measured using a Bio-Rad DC colorimetric protein assay kit. Serial dilutions of the sample and a protein standard (BSA, Bio-Rad) were prepared by mixing with lysis buffer. 5 µl of the diluted samples and the BSA standards were pipetted into a 96-well plate in a triplicate. 25 µl of working reagents, prepared by mixing 60 µl reagent S and 3 ml reagent A, were added to each plate, after that, 200 µl of reagent B were added to each plate. The plate was incubated for 15 minutes at room temperature. Finally, a standard curve was generated by reading the absorbance at 750 nm. After quantification, 30 µg protein of each sample were stored at -20°C until further use. **Figure 2.3** represents an example of a generated standard curve in this project.

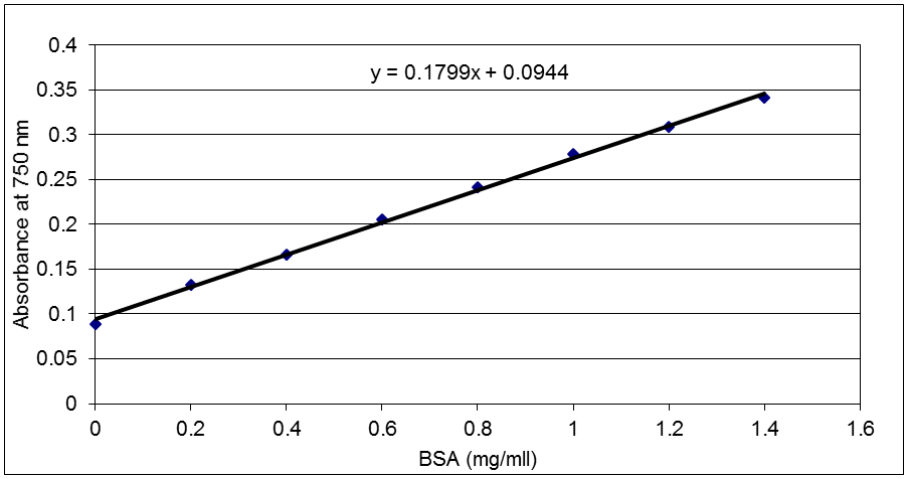

Figure 2.3. Typical protein concentration standard curve

The x axis of the graph represents protein concentration. The y axis represents the mean absorbance at 750 nm.

2.6 Western blotting

This is a classical approach to detect protein expression in a biological sample based on an immunological reaction (Towbin, Staehelin et al. 1979). The method was first reported by Southern in 1975 (Southern 1975).

To prepare the samples, 2 x Laemmli buffer (6% glycerol, 1.5 % SDS, and 10 Mm Tris HCL pH 6.8) was used to denature the lysate, and it was prepared by mixing 900 µl of Laemmli buffer + 100 µl of B-mercaptoethanol in a fume hood. After that, 30 µg of the lysate were mixed with 30 µl of the Laemmli buffer. The resulting suspension was stored at -20°C.

A low concentration acrylamide gel was used to separate PC1 protein because of its high molecular weight, while lower molecular weight proteins (PC2, ErbB4 and TSG-101) were separated using higher concentration acrylamide gels. 6% separating gel was prepared by mixing 9 ml H_2O, 4.0 ml 1.5 M Tris pH 8.8, 2.7 ml acrylamide 30%, 160 µl SDS 10%, 160 µl APS 10% and 16 µl TEMED. 10% separating gel was prepared by mixing 6.3 ml H_2O, 4.0 ml 1.5 M Tris pH 8.8, 5.4 ml acrylamide 30%, 160 µl SDS 10%, 160 µl APS 10% and 16 µl TEMED. A rack was assembled for gel solidification. The separating gel was poured and air bubbles removed by adding isopropanol on top of the separating gel. A 4% stacking gel was prepared by mixing 3.05 ml H_2O, 1.25 ml 0.5M Tris-HCl pH 6.8, 0.67 ml acrylamide 30%, 50 µl SDS 10%, 50 µl APS 10% and 12 µl TEMED. The mixture was poured on the top of separating gel. Simultaneously, a

comb was inserted to the stacking gel to make the wells. The SDS-gel was set up in an electrophoretic tank, and the tank was filled with a running buffer (1.5g Tris, 7.2 g Glycine, 0.5 g SDS and H_2O up to 500 ml). After that, the comb was removed, and 5 µl of a molecular weight marker (Bio-Rad, standard protein dual colour, cat no; 161-0373) and an equal concentration of lysate (30 µg) were loaded into each well. The gel was run at 100 V until the dye front had run off the bottom of the separating gel.

One polyvinylidene fluoride membrane (PVDF) and 2 filter sheets were cut to the same dimension of the gel. Before use, the PVDF membrane was activated in methanol for 2 minutes; then it was washed with distilled H_2O for 30 minutes. The gel was retrieved from the glass plates. The gel, 2 sponges, 2 filter sheets and the PVDF membrane were soaked in a transfer buffer (3 g Tris, 14 g glycine, 200 ml methanol and 800 ml distilled H_2O). A transfer sandwich was created as follows; sponge; 1 filters paper; gel; PVDF; 1 filter paper, sponge (**Figure 2.4**). The sandwich was inserted into a cassette and the transfer tank was filled with transfer buffer, and an ice bag was put in the tank to maintain the temperature at 4°C. The gel was transferred at a constant voltage (100 V) for 60 minutes.

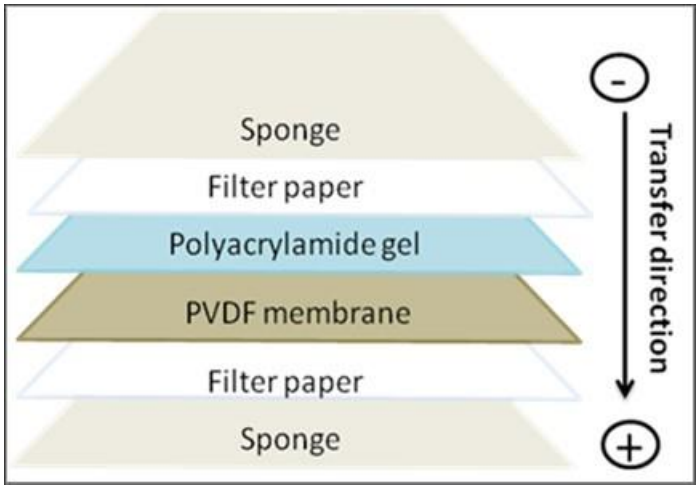

Figure 2.4. Assembly of western blot transfer sandwich

After western blotting the PVDF membrane was blocked with 10% skimmed milk in TBST (40 g NaCl, 50 ml Tris PH 7.6, 10 ml Tween 20 and up to 500 ml H_2O) for 1-3 hours at 4°C while shaking.

Immunoblotting was performed using specific antibodies. The primary antibodies and their dilutions for this project are summarised in **Table 2.3**. Primary antibodies were diluted in 5% skimmed milk in TBST, and incubated with the membrane overnight at 4°C with gently shaking. Following overnight incubation, the membrane was washed with TBST every 10 minutes for 60 minutes while shaking at room temperature. Next,

the membrane was incubated with diluted secondary antibodies in 5% skimmed milk in TBST for 60 minutes at room temperature. Then, the membrane was washed with TBST 6 times every 10 minutes at room temperature.

After that, the membrane was incubated with chemiluminescence substances (Bio-Rad, Clarity™ Western ECL substance) to develop the membrane. The membrane was placed on a plastic membrane and covered with the substance, by mixing 1 ml of each bottle (Paroxide solution and Luminal/enhance solution), and was left for 5 minutes, and after that, the excess volume of the substance was wiped. Finally, the membrane was exposed to a molecular imager, Bio-Rad ChemiDoc™XRS.

Table 2.3. Primary and secondary antibodies used for western blotting

Type	Directed against	Dilution	Source	Catalogue number
Primary antibodies	PC1 (Mouse monoclonal antibody, 7e12)	1;3000	Kidney Genetics Group	(Ong, Harris et al. 1999)
	PC2 (Mouse monoclonal IgG_{2b})	1;1000	Santa Cruz Biotechnology	SC-28331
	TSG-101 (Mouse monoclonal antibody)	1;1000	Abcam	Ab83
	ErbB4 (Rabbit monoclonal antibody)	1;200	Abcam	Ab76303
	THP (Rabbit polyclonal antibody)	1;5000	Santa Cruz Biotechnology	SC-20631
	AQP-1 (Rabbit polyclonal antibody)	1;1000	Santa Cruz Biotechnology	Sc-28629
Secondary antibodies	PC1 (Goat-anti-mouse IgG1-HRP (MIgG1))	1;5000	Southern Biotech	1070-05
	AQP1, ErbB4 and THP (Polyclonal Goat-Anti-Rabbit / HRP)	1;5000	Dako	P0448
	TSG-101 and PC2 (Polyclonal Goat-Anti Mouse / HRP)	1;5000	Dako	P0447

The table summarizes the marker used for western blotting, source, catalogue number and their concentrations.

2.7 Retrospective analysis of historical samples

Urine specimens were selected from a Sheffield Kidney Institution (SKI) bio-repository. These samples were obtained from an ongoing study into predictive biomarkers of disease progression in chronic kidney disease from patients with diabetic nephropathy and non-diabetic nephropathies (collected 2009-2010). Out of 400 urine specimens, 20 samples were initially selected for analysis in this project; 5 samples from each group were pooled together (5 ADPKD patients, 5 DM patients, 5 HTN patients and 5 healthy controls).

2.8 Cell free urine

2.8.1 MicroRNAs extraction from cell free urine

MicroRNAs extraction from cell free urine was performed according to the Exiqon recommended protocol using the miRCURY biofluids RNA extraction kit (cat no; 300112). In brief, starting urine volume was 200 µl. For the purpose of microRNAs stabilization during a reverse transcription step, bacteriophage MS2 (RNA carrier) was used, as it is free of microRNAs (provided by Roche Applied Science). Prior to isolation, 1 µl of MS2 was added per sample to miRCURY lysis solution followed by vortexing. At the same time, 1 µl of spike-in microRNA (UniSp2, UniSp4 and UniSp5) was added per sample to the lysis solution, followed by vortexing. After that, 60 µl of lysis solution was added to the resulting supernatant urine. Proteins were precipitated by adding 20 µl of protein precipitation solution followed by vortexing. The mixture was incubated for 1 min at room temperature and spun down for 3 minutes at 11,000 x g. The clear supernatant was transferred into a new collecting tube and 270 µl of isopropanol was added followed by vortexing for 5 seconds.

MicroRNA mini spin column was placed in a collection tube and the sample was loaded into it. Then, the column was incubated for 2 minutes at room temperature. The column was centrifuged for 30 seconds at 11,000 x g, and then, the flow-through was discarded. 100 µl of wash solution-1 was added to the microRNA column and followed by centrifuging for 30 seconds at 11,000 x g, and then, the flow-through was discarded. Two washing steps with wash solution-2 were performed, 700 µl and 250 µl respectively. After each wash, the column was spun down and the flow through was discarded. Elution of microRNAs from the column was carried out by adding 50 µl of RNase free water followed by incubation for 1 min at room temperature. After that, the

column was centrifuged at 11,000 x g for 1 min. Finally, the microRNAs were aliquoted into RNAase free tubes (10 µl each), and stored at -80°C until further use.

2.8.2 Reverse transcription (RT)

First strand cDNA was synthesised using miRCURY LNA™ microRNA PCR universal cDNA synthesis kit II (cat no; 203301). Prior to commencing this step, the working area was cleaned with RNAase away solution (provided by Invitrogen life technology). All provided reagents and the microRNA templates were thawed on ice except for an enzyme mix which was removed from the freezer immediately before use. All reagents were spun down before use. RT reaction solution was prepared by mixing 5 x reaction buffer, nuclease free water, enzyme mix, synthetic RNA spike-ins and microRNAs template in nuclease free tubes according to **Table 2.4** protocol. The reaction solution was mixed by gentle vortex followed by spin down. In order to perform reverse transcription, the thermal cycler was programmed as described in **Table 2.5**. The resulting cDNA was stored undiluted at -20°C.

Table 2.4. Reverse transcription reaction

Reagents	96 well plate (µl)	384 well plate (µl)	Individual assay (µl)
5 x reaction buffer	4	8	2
Nuclease free water	9	18	4.5
Enzyme mix	2	4	1
Synthetic RNA spike-ins (Unisp-6 and cel-miR-39-3p)	1	2	0.5
Template microRNAs	4	8	2
Total volume	20	40	10

The table shows the total RT working solutions for different assays; 96 well plate for the quality control, 384-well plate for the discovery phase and individual assays for the validation assays.

Table 2.5. Reverse transcription thermal cycler

Temperature (ºC)	Time (minutes)
42	60
95	5
4	Hold

2.8.3 Quantitative PCR amplification

According to the type of the assay, known volumes of PCR master mix, PCR primers, cDNA templates and a passive reference dye (Rox) (1 µl for each 50 µl RT mixture) were mixed together according to the **Table 2.6**. 10 µl of qPCR reagents mix were loaded to quality control plate (Exiqon microRNA 96 well QC PCR Panel, V4.ST, cat no; 203890), this panel contains spike–ins and the most common microRNAs in urine, or to 2 x 384 well plate array (human panel I+II, V3.M, cat no; 203611) contain a total of 752 pre-defined microRNA probes, or to the individual assays for the validation assays.

Table 2.6. Quantitative PCR reaction mix

Reagent	96 well plate (µl)	384 well plate (µl)	Individual assay (µl)
PCR master mix	70	2000	5
PCR primer mix	Preloaded to the plate	Preloaded to the plate	1
Undiluted cDNA template	1.4	40	16 µl of diluted cDNA (382 water + 4 µl cDNA + 8 µl Rox dye)
Nuclease free water	65.8	1880	/
Rox reference dye	2.8	80	/
Total volume	140	4000	10

The table shows the total qPCR working solutions for different assays; 96 well plate for the quality control, 384 well plate for the discovery phase and individual assays for the validation assays.

In order to reveal any potential risk of contamination in the experiments, a non-template control (NTC) was prepared. NTC is a negative control which contains all reagents except the cDNA template which was replaced with nuclease free water. With the aim of reducing the risk of pipetting variation; a robust pipetting machine (Beckman coulter, Biomek^{R}NX^{P}) was used. The plate was sealed with an optical sealing (adhesive cover) and spun down in a plate centrifuge at 15,00 x g for 1 min. After that, the plate was loaded into a qPCR analyser machine (ABI 7900 HT). A thermal cycler protocol was set according to Exiqon protocol as shows in **Table 2.7** for 40 cycles**,** followed by a melting curve cycles as shown in **Table 2.8**.

Table 2.7. Quantitative PCR thermal cycler

Temperature (ºC)	Time (minutes)
95	10
95	10
60	1

Table 2.8. Melting curve cycles

Temperature (ºC)	Time (seconds)
95	15
60	15
95	15

2.8.4 Data analysis

Raw data was exported from an ABI 7900 machine and imported into DataAssist software (version 3.01, Applied Biosystems) as a text file. The relative levels of urinary microRNAs were presented as threshold cycle values (Ct value). Delta Ct values (CT value of a microRNA - CT value of a selected spike-in) were used to measure the expression of a microRNA in a given sample. UniSp3 was used as a control for urinary microRNAs discovery phase as recommended by Exiqon; whereas UniSp 2, 4 and 6 spike-ins were used for the validation experiments.

2.9 Prospective study

Urine specimens were selected from our PKD cohort to match for age and gender with healthy controls for the following experiments.

2.10 Exosomal associated microRNAs extraction

Urine exosomal associated microRNAs were isolated from an exosome pellet isolated using a miRCURY™ RNA cell and plant isolation kit (cat no; 300110) (Lasser, Eldh et al. 2012). The protocol consists of 4 basic steps; the first step was to lyse the exosome pellet with 350 µl of provided lysis buffer. After that, 200 µl of 95% ethanol was added to the lysate. The mixture was then vortexed for 10 seconds. The third step was to assemble Exiqon' mini column with a provided collecting tube and 600 µl of the mixture was added to the column. The filter column was centrifuged at 14,000 x g for 1 minute and the flow through was discarded. After that, the mini column was washed 3 times with 400 µl of washing solution. The final step was microRNAs elution, with 50 µl of elution buffer followed by centrifuge at 200 x g for 2 minutes, then at 14, 000 x g for 1 min. The purified microRNAs were stored at -80^{o}C until use.

2.11 Concentration of microRNA yield

Because of the anticipated low yield of microRNAs from urine exosome samples, ammonium acetate was used to concentrate the resulted RNA. The protocol of RNA concentration is displayed in **Figure 2.5**.

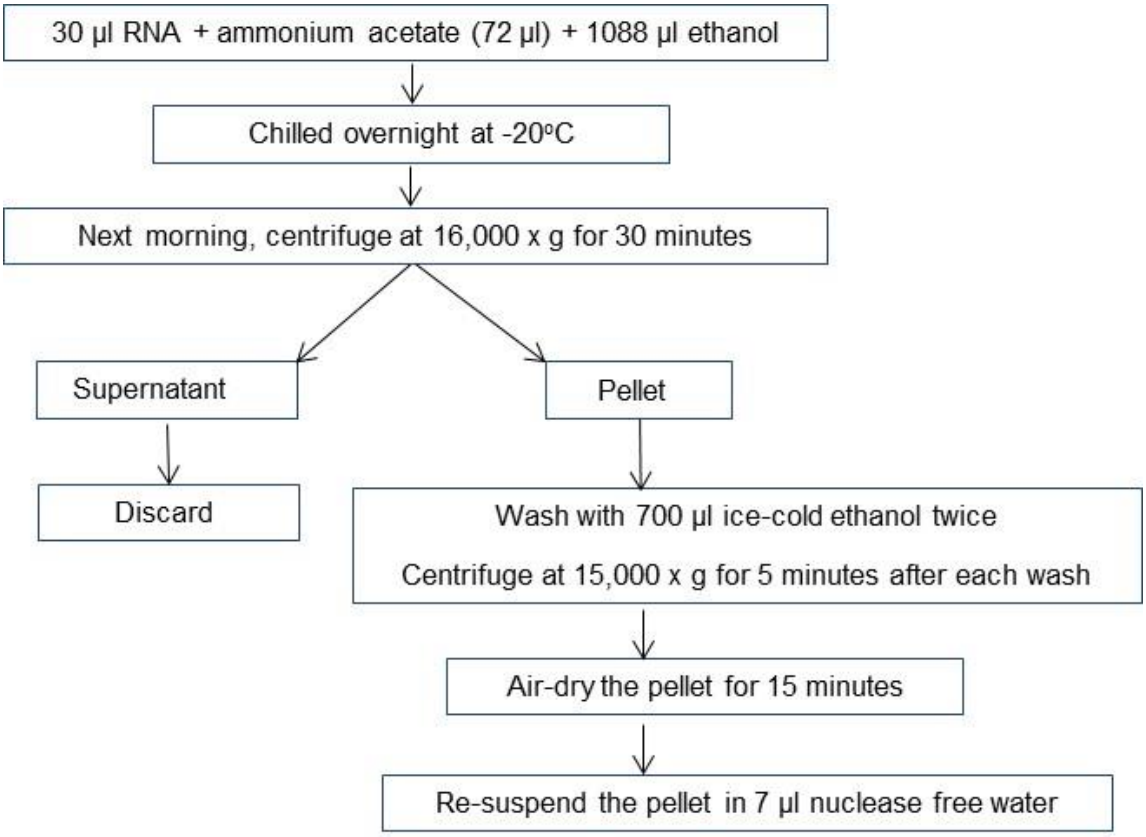

Figure 2.5. Maximization of exosomes associated RNA using RNA concentration technique

2.12 Small RNA library preparation for next generation sequencing

Small library preparation for next generation sequencing was conducted in collaboration with Mr Mathew Wyles based in the Sequencing facility, SITran, University of Sheffield, using TruSeq® Small RNA library preparation kit (cat no; RS-2009002DOC, Illumina). Before proceeding with small RNA library preparation, the initial suspended RNA volume (6 µl) was concentrated to 2.5 µl using a DNA concentrator machine (GeneVac). The work flow of small RNA library preparation for next generation sequencing is shown in the **Figure 2.6**.

2.12.1 Ligation adapters

The sequential ligation of the 3' and 5' RNA adapters to the sample were prepared according to TruSeq® Small RNA library preparation Illumina protocol. The Illumina consumables were removed from -20ºC and thawed on ice. After that, the consumables were centrifuged briefly at 600 x g for 5 minutes and then were placed back on ice.

Firstly, RNA3' adapter ligation, an adapter reaction was set up in a 200 µl nuclease free tube according to the volumes in **Table 2.9** on ice, and then incubated on a preheated thermal cycler for 2 minutes at 70°C. In a separate 200 nuclease free tube, a ligation reaction was prepared by mixing the volume in the **Table 2.10** on ice. 2 µl of the mixture was added to the adapter reaction tube and mixed thoroughly. Next, the tube was incubated on the preheated thermal cycler at 28°C for 1 hour. After that, 0.5 µl of Illumina stop solution to the reaction tube on the thermal cycler and pipetted up and down. The solution was incubated at 2°C for 15 minutes and then was immediately placed on ice.

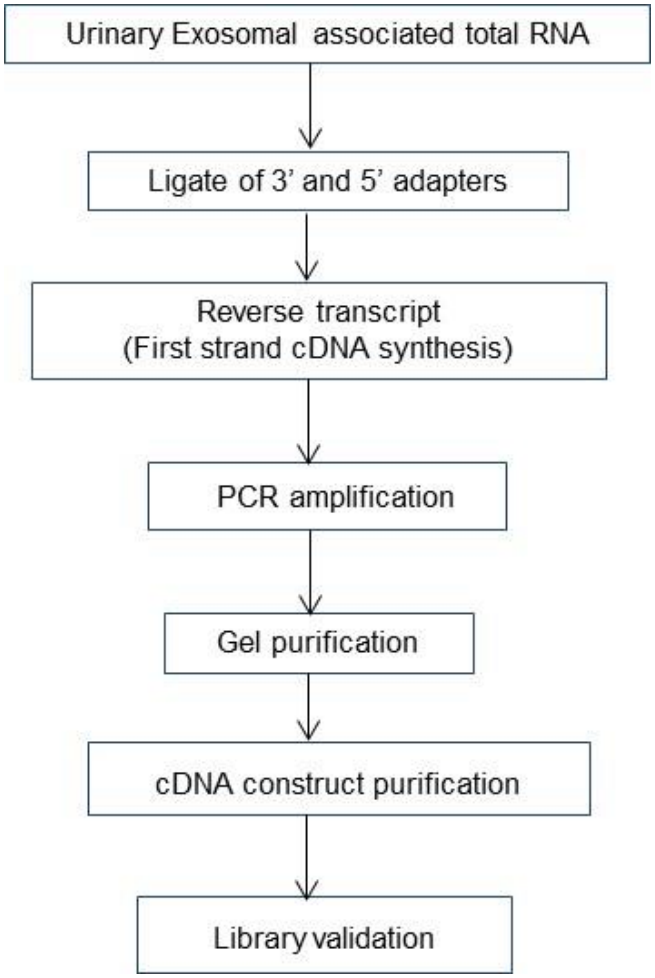

Figure 2.6. The work flow of small RNA library preparation

Table 2.9. Adapter reaction

Reagent	Volume (µl)
RNA3' adapter	0.5
Total RNA in nuclease-free water	5.5
Total volume	6

Table 2.10. Ligation reaction

Reagent	Volume (µl)
Ligation buffer	1
RNase inhibitor	0.5
T4RNA ligase 2, deletion mutant	0.5
Total volume per sample	2

The second step was RNA5’ adapter ligation, 0.6 µl of RNA5' Adapter (RA5) multiplied by the number of RNA samples being prepared was added to a nuclease free tube. The RA5 tube was placed on a preheated cycler at 70°C for 2 minutes and then placed immediately on ice. 0.6 µl of Illumina 10 mM ATP multiplied by N (the number of prepared samples) was added to the RA5 tube. Then, the mixture was pipetted up and down to mix thoroughly. Illumina T4 RNA ligase (1.5 µl X N µl) was added to the RA5 tube and then, gently mixed. 3 µl of the RNA 5' Adapter tube was added to the reaction from the ligation 3' adapter. The mixture was then pipetted up and down gently; and incubated on a preheated thermal cycler for 1 hour at 28°C, after that, the tube was placed on ice for the following step.

2.12.2 Reverse transcription (RT)

The reverse transcription step selectively enriches the RNA fragments with 3' and 5' adapters. This step was performed with 2 primers which specially annealed to the 3' and 5' adapters. The Illumina cDNA kit was thawed on ice, and the thawed consumables were briefly centrifuged for 5 seconds at 600 x g. Before preparation of the RT reaction, 25 mM dNTPs was diluted by mixing 0.5 µl of 25 mM dNTPs mix and 0.5 µl ultra-pure water in a separate nuclease free tube; this volume was multiplied by N (the number of

samples) + 10% extra reagents of the recommending volume. The diluted volume was gently pipetted up and down followed by a brief centrifuge and then placed on ice. In a 200 µl nuclease free tube, 6 µl of adapter-ligated RNA was mixed with 1 µl of RNA RT-primer followed by gently pipetting and a brief centrifuge. The tube was then incubated on a preheated thermal cycler at 70°C for 2 minutes and immediately placed on ice. At the same time, reagents in the **Table 2.11** were mixed in a separate PCR tube according to the volumes in the table (multiplied by N + 10% extra reagents). The entire volume was gently pipetted up and down, and then was centrifuged briefly. 5.5 µl of the reagent mixture was added to the previously prepared RNA and RNA RT primer tube. The mixture was gently pipetted up and down followed by a brief centrifuge. Finally, the mixture tube was placed on a preheated thermal cycler at 50°C for 1 hour and then immediately placed on ice. The total volume in the tube was 12.5 µl.

Table 2.11. Reverse transcription master mix

Reagent	Volume (µl)
5 X first strand buffer	2
12.5 mM dNTPs mix	0.5
100 mM DTT	1
RNase inhibitor	1
Super script II reverse transcriptase	1
Total volume per sample	5.5

2.12.3 Quantitative PCR amplification

Illumina kit contains 48 different indexed primers. Only 1 out of the 48 primers is used per sample. In a separate nuclease free tube for each primer index, the reagents in the **Table 2.12** (multiplied by N + 10% extra reagents) were mixed together, and placed on ice. 37.5 µl of the PCR master mix was added to the reaction tube from a reverse transcription tube and gently mixed by pipetting up and down followed by a brief centrifuge. The resulting mixture volume was 50 µl. Finally, the mixture was incubated on a thermal cycler as shown in **Table 2.13**.

Table 2.12. PCR master mix

Reagent	Volume (µl)
Ultra-pure water	8.5
PCR mix (PML)	25
RNA PCR primer (RP1)	2
RNA PCR primer Index (RPIX)	2
Total volume per sample	37.5

Table 2.13. Reverse transcription thermal cycler

Temperature (ºC)	Time (seconds)
98	30
98 (for 11 cycles)	10
60	30
72	15
72	10
4	Hold

2.12.4 Assessment of cDNA result

As recommended by Illumina, each cDNA library must be evaluated for the size distribution and the concentration of the final product using an Agilent Bioanalyzer. The Agilent Bioanalyzer is a capillary electrophoresis based technique which illustrates nucleic acid size distribution compared to a standard ladder. Based on the Agilent result, the average size and concentration of the final library can be determined before proceeding with next generation sequencing analysis.

After PCR amplification, 1 µl of each cDNA sample was run on an Agilent Bioanalyzer chip. Illumina provided an example for the expected result, total RNA extracted from human and mouse tissues as shown in the **Figure 2.7**. However, incorrect identification of the ladder marker by the machine could lead to a shift of the

bands from sample to sample. In addition, the amplification blot could be variable based on amount of RNA input, tissue species and type.

2.12.5 Purified cDNA construct

During this step, small RNAs were selected based on their molecular size. This was conducted with a size exclusion gel using a trasilluminator reader. Once the desired size is isolated, the cDNA library is eluted, and then was concentrated using an ethanol precipitation approach. Libraries with unique primer indexes were pooled together before a gel purification step. 50 µl of the amplified cDNA library was mixed with 10 µl DNA loading dye and loaded into a 6% Novex TBE gel and also custom RNA and high resolution ladders were loaded to the gel. The gel was run at 145 V for 1 hour. After finishing the run, the gel was removed from the electrophoresis unit.

2.12.6 Recovery of purified construct

After removing the gel, the gel was stained with ethidium bromide (0.5 µg/ml in water) in a clean container for 3 minutes. Then, the gel was viewed on a dark reader transilluminator, and 2 bands (147 nt and 157 nt bands) were taken in a single slide according to their molecular weight against RNA customer ladder using a razor blade (**Figure 2.8**). The 147 nt band represents 22 nt mature microRNA while the second band was a 157 nt represents 30 nt small RNA. The extracted band was placed into a gel break tube (0.5 ml) and then centrifuged for 2 minutes at 20, 000 x g. Ultra-pure water (200 µl) was added to the tube to be sure all of the gel passed through the hole. The eluted DNA was shaken at room temperature for around 2 hours. Finally, the eluted DNA was transferred to the top of a 5 µm filter and then, the filter was centrifuged at 600 x g for 10 seconds.

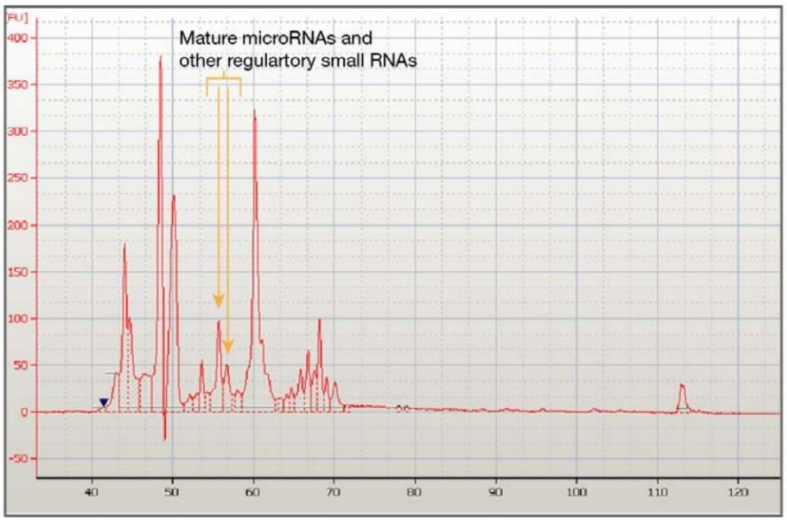

Figure 2.7. Amplification trace of human brain RNA sample on an Agilent Bioanalyzer

The graph shows an example of expected result of final library size distribution and concentration determined by an Agilent Bioanalyzer (Illumina small RNAs sequence datasheet).

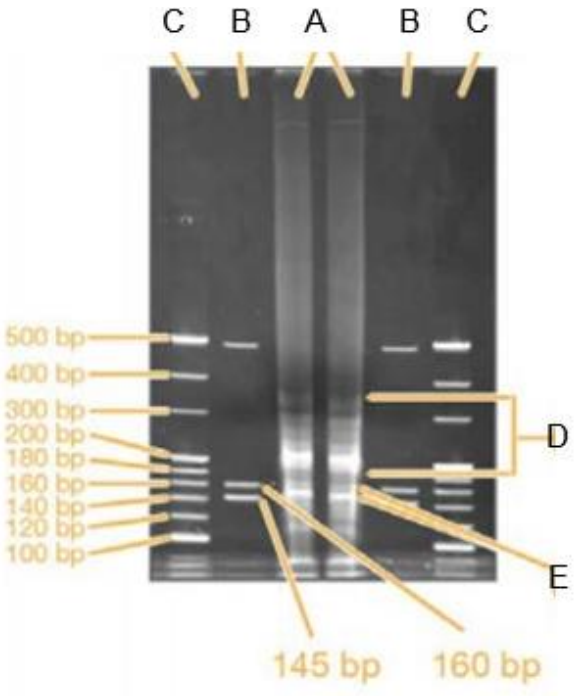

Figure 2.8. Gel electrophoresis of a purified cDNA construct library

The lanes are; (**A**) Human brain total RNA (**B**) custom RNA ladder (**C**) high resolution ladder (**D**) small noncoding RNAs (**E**) microRNA. D and E bands are purified cDNA construct of human brain total RNA (Illumina small RNAs sequence datasheet).

2.12.7 Final library concentration

After the gel electrophoresis, the filter was discarded and 975 µl of pre-chilled 100% ethanol, 30 µl 3M NaOAc and 2 µl glycogen were added to the RNA elutes. Next, the mixture was centrifuged for 20 minutes at 20,000 x g at 4°C, the supernatant was discarded. The final pellet was washed with a 70% ethanol and then centrifuged at room temperature for 2 minutes at 20,000 x g. The resulting supernatant was discarded while the pellet was dried by heating at 37ºC for 5-10 minutes. Finally, the pellet was re-suspended in a 10 µl of 10 mM Tris-HCI, pH 8.5.

2.12.8 Library validation

Before proceeding to a cluster generation, library validation was conducted to ensure accurate quantification of the library. Every peak on the Bioanalyzer creates a cluster and the final molarity is the result of summation of all peaks on the electropherograms. To do that, 1 µl of the constructed library was loaded on an Agilent Bioanalyzer. **Figure 2.9** shows an example of the Agilent Bioanalyzer check using total RNA of human tissue.

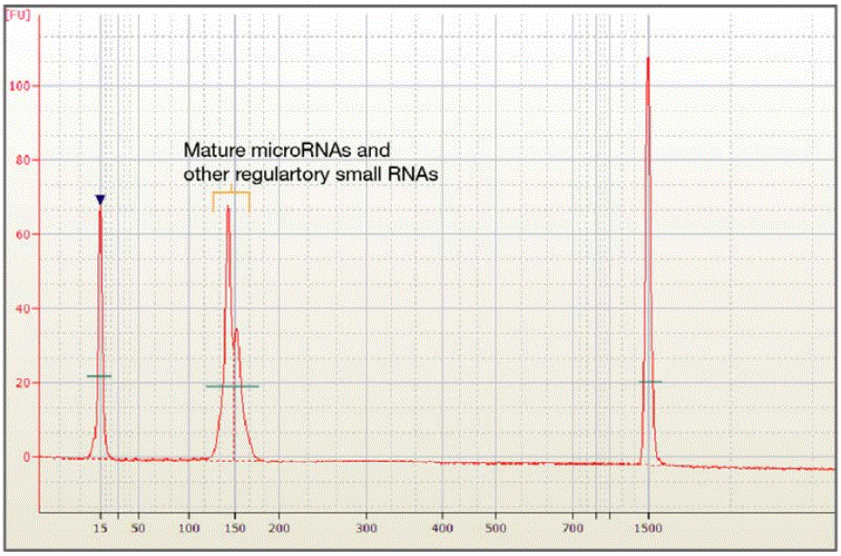

Figure 2.9. Small RNA library validation

2.12.9 Sequencing

The machines were operated entirely by Mr Mathew Wyles. Ligated cDNA fragments were amplified in the flow cells of Illumina cBoot machine. Every flow cell contains 8 channels which coated with complementary nucleotides to the ligation adapters. The ligated cDNA fragments were hybridized to the channels and amplified to form a bridge and clustering together. Each cluster contains approximately 1000 copies of the sample DNA (Berglund, Kiialainen et al. 2011). TruSeq SR (single read) cluster kit (V3, cat no; GD-401-3001) was used for cBoot cluster generation. Before proceeding with cluster generation, normalization of the libraries was performed to 2 nM using of Tris-HCl 10 mM, pH 8.5. The cluster generation protocol began with denaturation of DNA template to a final concentration of 20 PM to optimize the hybridization step.

Briefly, 2 nM template DNA (10 µl) and 0.1 NaOH (10 µl) were added to a centrifuge tube followed by a brief vortex, and then was centrifuged for 1 min at 280 x g. To denature the templates, the mixture was incubated at room temperature for 5 minutes, then, 20 µl of the denature template was transferred to a nuclease free tube containing 980 µl of a Hybridization Buffer (pre-chilled HT1) and placed on ice. The final dilution of the denatured DNA was prepared by mixing of 750 µl denatured DNA and 250 µl of pre-chilled HT1 to achieve a concentration of 15 pm. The mixture was mixed by inverted the tube several times followed by a brief centrifugation. The diluted denature was placed on ice until running the samples on the cBoot machine. 120 µl of template DNA was loaded into an 8 tube stripe and loaded to the machine. Finally, the clustering flow cells were loaded for sequencing using Illumina sequencing instrument (HiScan SQ).

2.12.10 Bioinformatics analysis

The initial stage of data analysis was to export the sequenced data from the Illumina sequencing instrument and then was uploaded to bcl2fastq conversion software, V1.8.4,(https://support.illumina.com/downloads/bcl2fastq_conversion_software_184.html). The data was de-multiplexed according to their specific barcode code into FASTQ format. Differential microRNA expression analysis was then carried out using 3 separate software packages.

Strand NGS differential expression analysis

The sequenced data was imported as FASTQ files to Strand NGS, version 2.5.1 (Strand Life Sciences, Bangalore, India), (http://www.strand-ngs.com/). Data analysis was started by creating a small RNA analysis experiment. Default parameters were used for alignment quality controls and for further data analysis steps. Small RNA alignment experiment was then created, using microRNA annotations taken from miRBase (version-21). Before proceeding to reads alignment, Illumina adapter sequence (5'TGGAATTCTCGGGTGCCAAGG) was stripped from the sequence reads. Reads were discarded if the adapter sequenced were absent: Data was aligned to human genome (hg19). The sequence read mapping was carried out according to the default settings. The number of mismatch allowed was specified at 1. The expression level of microRNA was normalized between samples using the default DESeq normalisation algorithm.

Chimira NGS differential expression analysis

Chimira is a small RNA sequencing analysis online pipeline, version 1.0 (Vitsios and Enright 2015). The sequence reads were uploaded and analysed directly on the web server, (http://wwwdev.ebi.ac.uk/enright-dev/chimira/). Analysis was run according to standard settings of the pipeline. Reads were mapped to human genome (hg19). All sequence reads are mapped against miRBase database, version-21 (Griffiths-Jones, Saini et al. 2008). The pipeline setting allowed up to 1 mismatch for each read. DESeq was used to normalize the sequencing reads. The pipeline calculates the differential expression levels of microRNAs between 2 samples or groups (Griffiths-Jones, Saini et al. 2008).

Illumina NGS differential expression analysis

The FASTQ files were imported into an Illumina BaseSpace pipeline for downstream analysis, (https://www.illumina.com/informatics/research/sequencing-data-analysis-management/basespace/basespace-apps.html). The pipeline offers different applications including small RNA, microRNAs and RNAs analysis. MicroRNAs application of BaseSpace was selected, and a FASTQ file for each sample was uploaded into a new project. With the aim of identifying differentially expressed microRNA, the samples were categorised into two groups (healthy control and ADPKD group) and then the application was launched to perform the analysis. DESeq was used to normalize the sequencing reads.

2.13 TaqMan-microRNA qPCR

2.13.1 MicroRNA reverse transcription (RT)

TaqMan microRNAs reverse transcription assay was used in these experiments (cat no; 4366596). Prior to the reverse transcription step, a cel-miR-39-3p spike-in was added to the RNA as an exogenous control. RT-master mix was prepared by combination of the volumes in the **Table 2.14** in a nuclease free tube. After that, the tubes were incubated in a thermal cycler (**Table 2.15**).

Table 2.14. Reverse transcription master mix

Component	Volume (µl)
dNTPs mix	0.15
MultiScribe™ reverse transcriptase	1
10 × reverse transcription buffer	1.50
RNase inhibitor	0.19
Nuclease-free water	4.16
Total	7

Table 2.15. Reverse transcription thermal cycler

Temperature (°C)	Time (minutes)
16	30
42	30
85	5
4	Hold

2.13.2 Quantitative PCR amplification

TaqMan universel PCR master mix was used in this step (cat no; 4324018). The qPCR amplification was prepared according to the volumes in **Table 2.16**. After that, the mixture was mixed gently. 10 µl of the reaction was transferred into PCR plate wells in triplicate. Then, the plate was run on an ABI 7900 machine according to the thermal cycler in **Table 2.17**. Finally, the data was analysed using DataAssist software (version 3.01, Applied Biosystems). Delta Ct values, CT value of a microRNA - CT value of a selected control, were used to measure the expression of a microRNA in a given sample. 2^{DDCt} of candidate microRNA ($2^{\text{Delta Ct (patients)} - \text{Delta Ct (controls)}}$) was calculated to measure a fold change of the microRNA expression in urine samples from the patients group relative to healthy controls group.

Table 2.16. Quantitative PCR reaction

Component	Volume / sample (µl)
TaqMan universal PCR master mix II	5
Nuclease-free water	3.83
TaqMan small RNA Assay (20×)	0.5
Product from RT reaction	0.67
Total	10

Table 2.17. Quantitative PCR thermal cycler

Temperature (°C)	Time	Run (cycles)
50	2 minutes	/
95	10 minutes	/
95	15 seconds	40
60	60 seconds	40

2.14 Gene expression

2.14.1 Reverse transcription (RT)

TaqMan high capacity RNA to cDNA kit was used in these experiments (cat no; 4387406) which contains 2 x RT buffer and a 20 X enzyme mix. The RT reaction was prepared according to the volumes in the **Table 2.18**. The reaction was aliquoted into tubes (according to the number of the samples), and placed on ice until use. After that, the tubes were incubated in a thermal cycler (**Table 2.19**), and the resulted cDNA was kept on ice until further use.

Table 2.18. Reverse transcription reaction

Component	Component volume / reaction (µl)	
	+ RT	- RT
2 x RT buffer	10.0	10.0
20 X enzyme mix	1.0	/
RNA sample	Up to 9	Up to 9
Nuclease- free H_2O	Sufficient to 20	Sufficient to 20
Total per reaction	20.0	20.0

Table 2.19. Reverse transcription thermal cycler

Temperature (^oC)	Time (minutes)
37	30
95	5
Hold	15

2.14.2 Quantitative PCR amplification

Quantitative PCR was carried out using TaqMan® Gene Expression (cat no; 4387406). The volume was mixed according to the volumes in **Table 2.20**. 10 µl of PCR reagents mix were loaded to a 378 well-plate in triplicate for each sample. Finally, the plate was run on an ABI 7900 machine according to the thermal cycler in **Table 2.17**. Data was analysed using DataAssist software (version 3.01, Applied Biosystems).

Table 2.20. Quantitative PCR reaction

Component	Component volume / reaction (µl)	
	Per well	triplicate
TaqMan gene expression master mix (2✕)	5	17.5
Primer	0.5	1.75
Diluted cDNA template (+H_2O: 1;1)	1.25	3.75
Nuclease- free H_2O	3.25	11.37

2.15 Angiogenesis proteome array profiling

To compare urinary expression of related angiogenesis factors between patients with ADPKD and healthy controls, a high throughput proteomic angiogenesis array was used; R&D human angiogenesis proteome array (cat no; ARY007). The proteomic array is a fast, sensitive technique to detect differential expression of angiogenesis and cytokine related proteins in urine (Liu, Zhang et al. 2006). Each array contains 55 angiogenesis-related proteins (**Figure 2.10** and **Table 2.21**).

5 urine samples (3 ml each) were pooled in each examined group. Pooled urine samples (15 ml) were concentrated to 2 ml by centrifuge at 4,000 x g for 30 minutes using Amico ultra, 3 kDa protein concentrations. The array was blocked with 2 ml of array buffer-7 on a rocking shaker for 1 hour. Samples were prepared by adding 1 ml of concentrated urine to 0.5 ml of array buffer. Reconstituted detection antibody cocktail (15 µl) was added to the prepared samples and incubated for 1 hour at room temperature. Following this, the array buffer-7 was aspirated from the wells and the sample/antibody mixture were added to each membrane and incubated overnight at 4^{o}C.

On the second day, the membranes were carefully removed and washed 3 times with a washing buffer on a shaker, 10 minutes each washing. Each membrane was carefully removed from its wash container and incubated for 30 minutes with diluted streptavidin-HRP. The washing steps were repeated as described above for a total of 3 times.1 ml chemiluminescence reagents 1 and 2 were mixed and added to each array. The arrays were covered carefully with a plastic sheet and gently smoothed out to remove any air bubbles. The membranes were then incubated with chemiluminescence reagents for 1 min. Finally, the membrane was exposed to a molecular imager, Bio-Rad ChemiDoc ^{TM}XRS for 10 minutes.

Individual signal densities were quantified using image lab software. The calculated pixel intensities then were exported into Microsoft Excel. The average densities of the pair of duplicate spots were determined. The calculated average densities of positive signals were subtracted from an average of background densities. The relative changes in the proteins expression between samples were compared and plotted using graphpad prism.

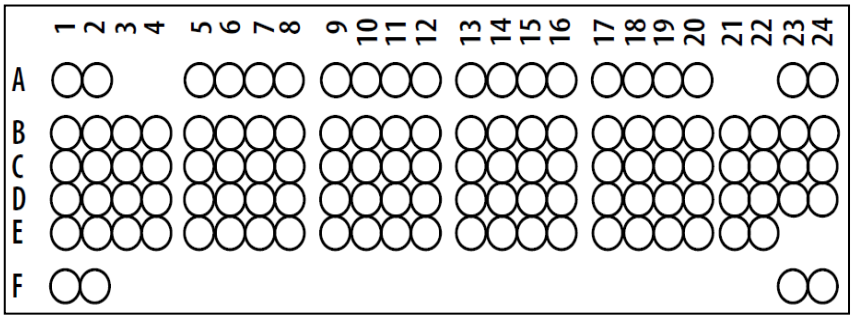

Figure 2.10. Human antibody array membrane

The figure shows the coordinate references for the angiogenesis related proteins. Table 2.20 shows protein names and reference spot (RnDSystems.com/ Proteome Profiler datasheet).

Table 2.21. Human angiogenesis array proteins

Coordinate	Protein	Coordinate	Protein
A1, A2	Reference spots	**C17,C18**	IL-8
A5,A6	Activin A	**C19,C20**	LAP(TGF-β1)
A7,A8	ADAMTS-1	**C21,C22**	Leptin
A9,A10	Angiogenin	**C23,C24**	MCP-1
A11,A12	Angiopoietin-1	**D1,D2**	MIP-1α
A13,A14	Angiopoietin-2	**D3,D4**	MMP-8
A15,A16	Angiostatin/Plasminogen	**D5,D6**	MMP-9
A17,A18	Amphiregulin	**D7,D8**	NRG1-β1
A19,A20	Artemin	**D9,D10**	Pentraxin3(PTX3)
A23,A24	Reference spots	**D11,D12**	PD-ECGF
B1,B2	Coagulation factor III	**D13,D14**	PDGF-AA
B3,B4	CXCL16	**D15,D16**	PDGF-AB/PDGF-BB
B5,B6	DPPIV	**D17,D18**	Persephin
B7,B8	EGF	**D19,D20**	PlateletFactor4(PF4)
B9,B10	EG-VEGF	**D21,D22**	PlGF
B11,B12	Endoglin	**D23,D24**	Prolactin
B13,B14	Endostatin/collagenXVIII	**E1,E2**	SerpinB5
B15,B16	Endothelin-1	**E3,E4**	SerpinE1
B17,B18	FGF acidic	**E5,E6**	SerpinF1
B19,B20	FGF basic	**E7,E8**	TIMP-1
B21,B22	FGF-4	**E9,E10**	TIMP-4
B23,B24	FGF-7	**E11,E12**	Thrombospondin-1
C1,C2	GDNF	**E13,E14**	Thrombospondin-2
C3,C4	GM-CSF	**E15,E16**	uPA
C5,C6	HB-EGF	**E17,E18**	Vasohibin
C7,C8	HGF	**E19,E20**	VEGF-A
C9,C10	IGFBP-1	**E21,E22**	VEGF-C
C11,C12	IGFBP-2	**F1,F2**	Reference spots
C13,C14	IGFBP-3	**F23,F24**	Negative control
C15,C16	IL-1β		

The table shows the coordinate references for the angiogenesis related proteins (RnDSystems.com/ Proteome Profiler datasheet).

2.16 Enzyme linked Immunosorbent Assay (ELISA)

In order to validate the results of angiogenesis discovery phase, ELISAs were selected for further validation of the deregulated proteins. In this project, sandwich based quantikine R & D systems ELISA kits were used. The sandwich ELISA principle is based on a microplate which had been pre-coated with a monoclonal antibody specific against human MCP-1 (cat no; DCP00), endostatin (cat no; DNST0) and prolactin (cat no; DPRL00).

Different dilutions of the urine samples (neat, 1;1, 1;2 and 10 x concentrated urine) were tested to identify the best dilution that gives a reading in the middle of the standard curve. For MCP-1 and endostatin, neat urine provided the best readings, whereas the concentrated sample provided the best reading in the case of prolactin. The latter is consistent with previous reports that urinary prolactin is detectable in concentrated urine (Keely and Faiman 1994). Therefore, a spot urine sample (10 ml) from each participant was centrifuged at 4,000 x g for 30 minutes to 1 ml using Amico ultra, 3 kDa.

Before commencing the protein assay, all reagents were brought to room temperature. Protein standard was reconstituted according to the manufactory recommendation. A dilution series of the candidate protein was generated by mixing protein standard with calibrator diluent of the assay into 8 tubes. Samples were loaded to the microplate in duplicate. In the case of prolactin and endostatin, 100 µl of assay diluent was added to each well. After that, a known volume of standard and samples per well were added to microplate and incubated at room temperature for 2 hours. Then, the microplate was aspirated and washed with provided washing solution (1;5 dilution) for a total of 4 times. After washing, excess fluid was removed by aspiration and decanting. Next, 200 µl of protein conjugate was added to each well and incubated. After 2 hours, plate washing for 4 times and aspiration were performed followed by incubation the plate with 200 µl of a substrate solution for 30 minutes. Then, 50 µl of a stop solution were added to each well. Finally, the optical density of each well was measured at 450 nm using a microplate reader. The concentration of the examined protein in the urine was calculated relative to the concentration of the standards.

2.17 Urine creatinine assays

Urine creatinine was measured for each participant by the biochemistry laboratory at the Royal Hallamshire Hospital, NHS trust, Sheffield. Java alkaline picrate method (an automated assay) was used to measure creatinine. The basis of the assay is as following; creatinine forms a yellow-orange colour in an alkaline solution and the rate of colour development is proportion to the concentration of creatinine in the urine sample. This technique has been standardized against IDMS traceable method. Cobas-8000 (701 models) machine was used to measure urinary creatinine. The assay range is 4.2 to 622 mg/dl.

2.18 Statistical analysis

Graphpad prism was used to analyse and express data (La Jolla California USA, version 6.05). Parametric data was analysed using independent T-test for 2 groups' comparison and one-way ANOVA for 3 groups followed by a Tukey's post hoc analysis; the results were expressed as mean ± SEM. Mann-Whitney U test was used to nonparametric data. P value < 0.05 was considered statistically significant. Spearman logistic regression was used to measure the correlations between the biomarkers and various clinical parameters.

SPSS (version 22) was used to calculate ROC curves, cut-off values, sensitivity and specificity of the examined biomarkers. A guide for interpreting the usefulness of a biomarker as a diagnostic test based on ROC curve is as follows: fail = o.5–0.6; poor = 0.6-0.7; fair = 0.7–0.8; good = 0.8–0.9; and excellent = 0.9-1 (Xia, Broadhurst et al. 2013).

Sample size was calculated using power calculation software. The sample size was calculated based on the rate of eGFR decline for ADPKD patients with progressive disease (> 3 ml/min/1.73m^2) using G power 3 (Faul, Erdfelder et al. 2007). A sample size of each chapter was calculated based on the **Figure 2.11**. For instance, a sample size of 58 patients provide 80% power to detect odd ratio of 2.2 (a relative measure of effective size) and type 1 error of 0.05 using z test (Demidenko 2007).

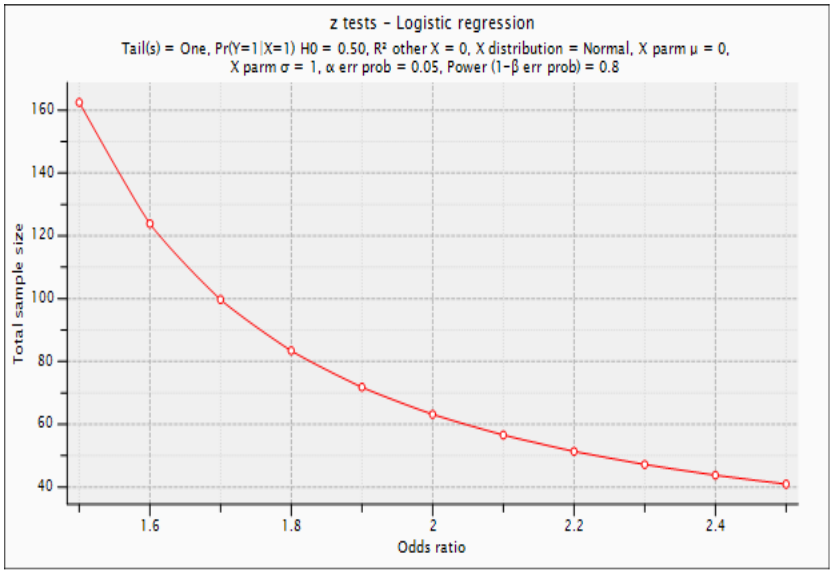

Figure 2.11. Sample size calculations

The x axis of the graph represents odds ratio. The y axis represents total sample size. This graph was generated using G power software based on eGFR slope > 3 ml/min over 5 years (Faul, Erdfelder et al. 2007).

Chapter 3 Exosome associated proteins

3.1 Introduction

Urine reflects pathophysiological alterations in the kidney and urological system, and is an attractive source of potential new protein biomarkers for clinical diagnosis and prognosis of renal diseases. However, the increased filtration of highly abundant proteins such as albumin could restrict its usefulness. The use of urine enriched exosomes may overcome this limitation (Pisitkun, Shen et al. 2004; Decramer, Gonzalez de Peredo et al. 2008).

ADPKD is a genetic disease and the potential role of exosome associated proteins in identification of genetic kidney diseases has been explored. For instance, Gonzales et al. examined the expression of exosomal associated sodium-potassium-chloride co-transporter-2 (NKCC2) in urine samples from patients with Bartter syndrome type I and healthy controls (Gonzales, Pisitkun et al. 2009). This study demonstrated an absence of NKCC2 in the patient's sample when compared to healthy controls.

ADPKD arises from loss-of-function mutations in *PKD1* and *PKD2*. Based on this observation, it could be predicted that a reduction in urinary expression of PC1 and PC2 or the detection of mutant PC proteins could be useful diagnostic or prognostic markers of disease. Interestingly, PC1 and PC2 bound to urinary exosomes (Pisitkun, Shen et al. 2004; Hogan, Manganelli et al. 2009) and mutant PC1 proteins observed in cystic cells derived from human *PKD1* patients with defined mutations (Ong, Harris et al. 1999; Qian, Boletta et al. 2002).

Recent work in our laboratory identified increased expression of ErbB4 in renal cystic tissue of human and mouse models of ADPKD using parallel microarray profiling study (Streets, Magayr et al. 2017). Of interest, ErbB4 has been detected bound to exosomes by other groups using proteomics analysis (Kim, Choi et al. 2012; Fraser, Moehle et al. 2013; Liang, Peng et al. 2013). Therefore, I decided to examine the expression of ErbB4 in ADPKD urine exosomes as a potential prognostic biomarker.

3.2 Hypothesis

Altered expression of urinary exosome associated PC1, PC2 and ErbB4 in ADPKD could be diagnostic or prognostic disease biomarkers.

3.3 Aim

The aim of this chapter was to examine if excretion of PC1, PC2, and ErbB4 in urinary exosomes were altered in patients with ADPKD compared to healthy controls, and could differentiate patients with slow and rapid disease progression.

3.4 Objectives

The main objectives to be addressed in this chapter were:

1. Optimize a method for purifying urinary exosomes and confirm their morphological and biochemical characteristics.

2. Quantify PC1, PC2 and ErbB4 expression levels in urine samples from patients with various stages ADPKD (eGFR > or < 60 ml/min/1.73m^2) and healthy controls.

3. Correlate candidate protein expression and structural or functional measures of renal function including eGFR, eGFR slope and mean kidney length (MKL).

4. Calculate the potential ability of these proteins to predict ADPKD progression.

3.5 Isolation and characteristics of urine exosomes

A key issue in the exosome field is the isolation method and the most widely used method is differential speed ultracentrifugation (Raposo, Nijman et al. 1996; Thery, Amigorena et al. 2006). In this project, exosome isolation was achieved by differential speed centrifugation of spot urine samples utilizing the Fernandez protocol (Fernandez-Llama, Khositseth et al. 2010). After the extraction, protein concentration was measured for each sample using a Bio-Rad DC colorimetric protein assay and 30 µg aliquots were stored at -20^{o}C until further use.

Exosomes originate from multivesicular bodies with specific characteristics which can be used to differentiate it from other types of secreted membrane vesicles (Bobrie, Colombo et al. 2011). Exosomes can be differentiated from other vesicles based on their size, shape and biochemical characteristics (Thery, Ostrowski et al. 2009). Based on vesicle size, exosomes are small round vesicles (40-100 nm), while apoptotic bodies are 1–5 µm in diameter, and the large vesicles are range from 100 to 1000 nm (Bobrie, Colombo et al. 2011; Gyorgy, Szabo et al. 2011; Urbanelli, Magini et al. 2013). Additionally, exosomes contain several unique proteins which allow their identification including tumour susceptibility gene-101 (TSG-101), alix and tetraspanins (Simons and Raposo 2009). To confirm the specificity of the purification method, electron microscopy and western blotting of exosome markers were conducted.

3.5.1 Biochemical characteristics of the isolated microvesicles

To confirm the origin of the isolated microvesicles, they were first analysed by western blotting. In brief, a spot urine sample from a healthy control was divided into four different aliquots: cell free urine, concentrated urine and 2 samples from each step of exosome isolation (exosomes depleted supernatant from 17,000 x g centrifugation and the final exosomes pellet) (**Figure 3.1**). 30 µg protein from each fraction of urine were loaded into a 10% SDS-PAGE gel. Next, western blotting was performed using antibodies against exosomal and non-exosomal protein markers including: mouse monoclonal antibody to TSG-101 (Abcam); Rabbit polyclonal to THP (Santa Cruz biotechnology, INC) and Rabbit polyclonal to AQP-2 (Santa Cruz biotechnology, INC).

Figure 3.2 indicates enrichment of exosome marker (TSG-101) in the purified microvesicles (Nabhan, Hu et al. 2012). AQP-2 was detected in the exosome fraction as well as the concentrated urine. THP was detected in all fractions of urine except exosomes depleted supernatant. THP is the most abundant protein in urine, and it's a

member of zona pellucid (ZP) domain family (Jazwinska and Affolter 2004). This family tends to form a polymeric meshwork between the ZP proteins by disulphide bonds (Fernandez-Llama, Khositseth et al. 2010). Therefore, THP polymerization traps the protein in the first 17,000 x g pellet and this may explain the lack of THP in the exosomes depleted supernatant fraction. Treatment with DDT disrupted the disulphide bonds and caused release of THP and other proteins into the supernatant of the second 17,000 x g centrifugation. These results demonstrate the efficacy of exosomes isolation using differential speed centrifugation.

3.5.2 Morphological characteristics of the isolated microvesicles

Electron microscopy permits assessment of the size and shape of the isolated microvesicles. After the extraction step, the exosome pellet was re-suspended in 50 µl 1% PBS and visualized using a Philips electron microscope 400 operated at 80 KV. The morphological features of exosomes were validated using electron microscopy which revealed small round vesicles with a diameter of 40-100 nm consistent with reported typical features of exosomes (**Figure 3.3**) (Thery, Boussac et al. 2001; van Niel, Raposo et al. 2001; Pisitkun, Shen et al. 2004; Kesimer, Scull et al. 2009).

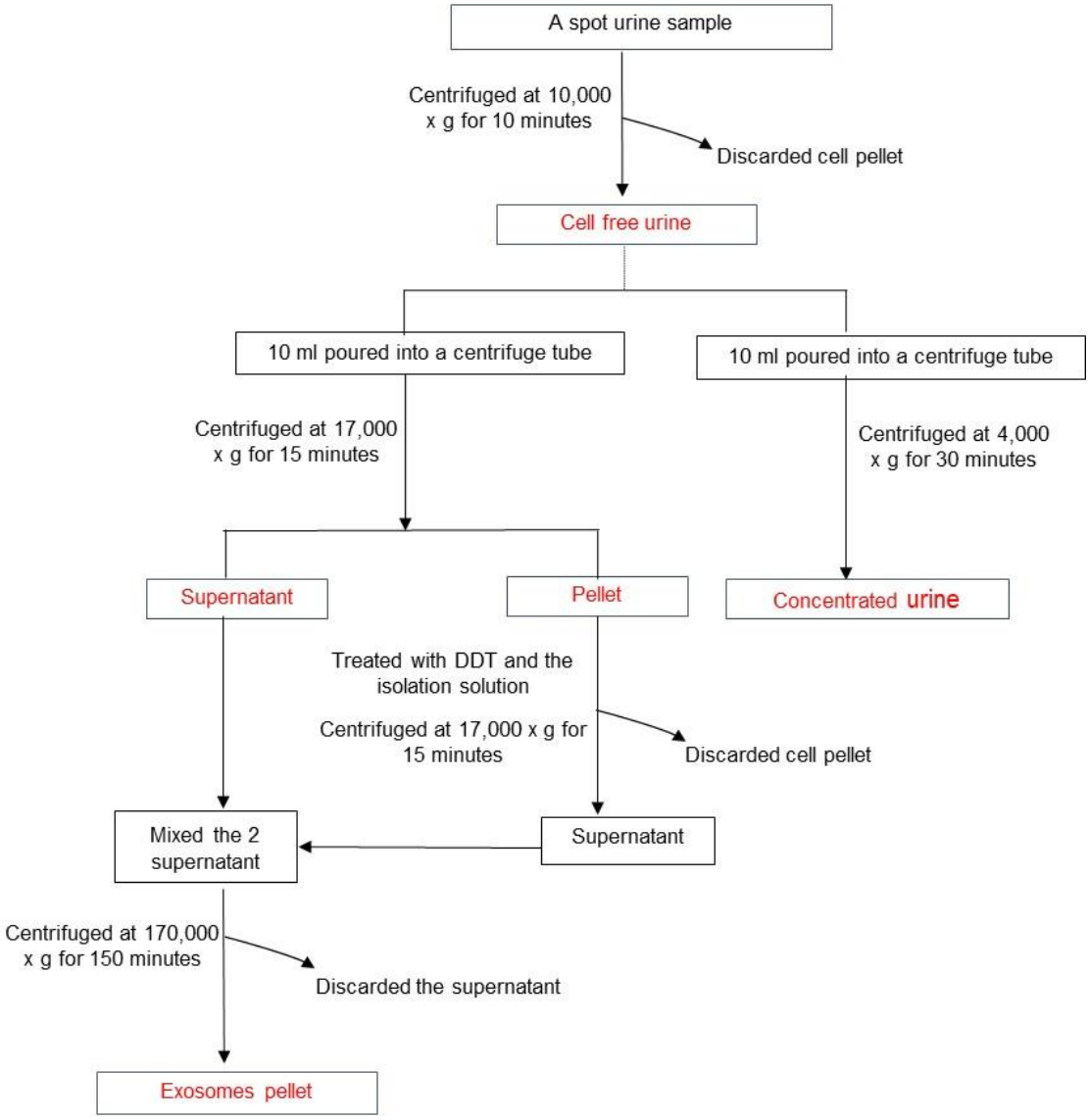

Figure 3.1. Flow chart of exosomes isolation

The figure shows the workflow of cell free, concentrated urine, exosomes depleted supernatant and exosome pellets isolation from a spot urine sample.

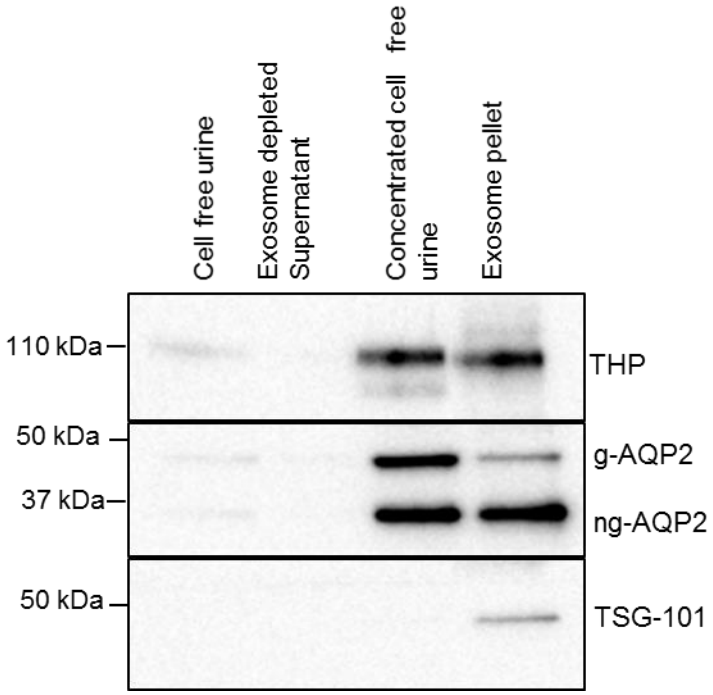

Figure 3.2. Biochemical characteristic of the isolated microvesicles

This blotting examined the presence of TSG-101, AQP2 (glycosylated and non-glycosylated) and THP in exosomes and non-exosomes urine fractions from a healthy volunteer. 30 µg protein from each fraction of urine were loaded into the gel. The TSG-101 blotting shows a clear band of ~50 kDa which was only detected in the exosomes pellet. The C-terminal of AQP-2 antibody recognized 2 bands: the glycosylated (35–45 kDa) and non-glycosylated (~29 kDa). Abbreviations are: THP; Tamm–Horsfall glycoprotein, AQP2; Aquaporin 2, TSG-101; Tumour Susceptibility Gene-101.

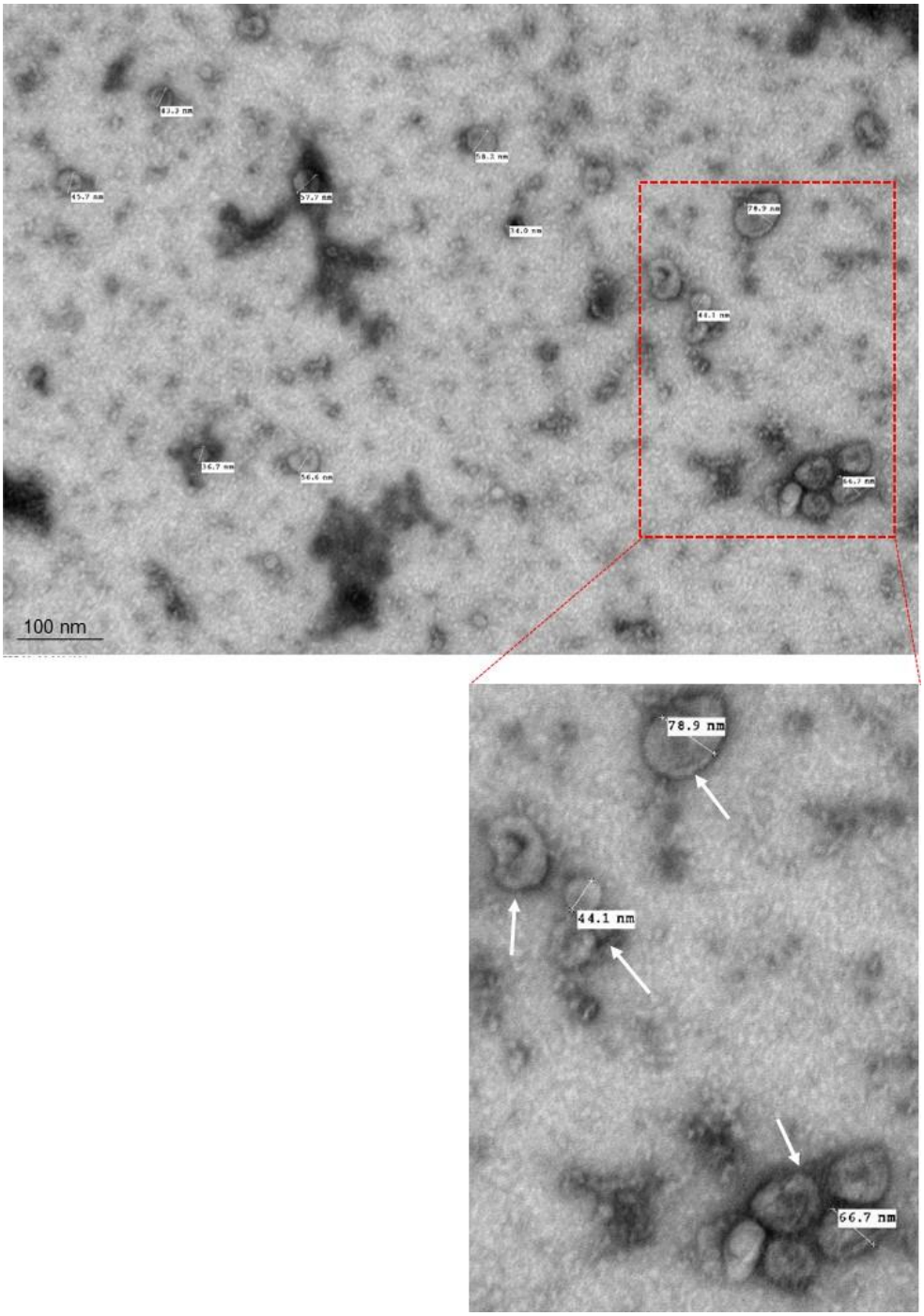

Figure 3.3. Electron microscopy of the isolated microvesicles

The figure shows isolated exosomes from a spot urine sample contrasted with 0.5% Uranium. Note the typical characteristic of exosomes as a small round cup-shaped microvesicles (indicated with white arrows). Scale bars = 100 nm.

3.6 Baseline characteristics of the study participants

Exosome associated PC1 and PC2 was examined in a cohort of 52 patients with ADPKD and 12 healthy controls. The sample size of 52 patients was calculated based on an eGFR slope > 3 ml/min over 5 years, and it provided 80% power to detect an odds ratio of 2.1 and type 1 error (alpha level) of 0.05 using z test.

The baseline clinical features of the participants are summarized in **Table 3.1**. Out of the 64 participants, 31 were males and 33 were females. Patients with ADPKD were subdivided into 2 groups based on their baseline eGFR at the time of recruitments: patients with eGFR > 60 ml/min/1.73m^2 (n = 26) and patients with eGFR < 60 ml/min/1.73m^2 (n = 26). The rate of renal function deterioration was also determined from the eGFR slope over 5 years. Patients with an eGFR decline of more than 3 ml/min/1.73m^2 over 5 years were categorised as having rapidly progressive disease while patients with a decline of less than 3 ml/min/1.73m^2 over 5 years were considered as having slowly progressive disease (Rosansky and Glassock 2014). Based on this definition, 25 patients were classified as rapid progressors and 27 patients as slow progressors.

The mean age ± SEM was 47.42 ± 3.652 in the control group, 44.38 ± 2.36 in the patients with eGFR > 60 ml/min and 56.19 ± 2.7 in the patients with eGFR < 60 ml/min; patients with eGFR < 60 ml/min were significantly older than the participants in the other 2 groups. There were significantly differences in the eGFR and uric acid levels at the baseline between the 3 groups. Among the two ADPKD groups, eGFR slope, mean kidney length (MKL) and PTH were significantly different; results of one-way ANOVA are shown in **Table 3.1**. There was no significant difference regarding gender, BMI, plasma cholesterol, serum calcium and serum phosphate between the groups. MKL of healthy controls was not measured in this project; however, previously published estimates are approximately 11.08 ± 0.96 cm (Kang, Lee et al. 2007). Kang et al. measured the MKL of a 125 living kidney transplant donors, out of them, 46 males and 76 females, with a purpose of use a different radiological measurement and body mass index as a predictor of renal length. That study found a difference in kidney dimensions between male and female; female kidney was smaller than male kidney by 0.2 ± 0.04 cm in length, 0.2 ± 0.40 cm in thickness and 0.2 ± 0.01 cm in width.

ErbB4 expression in urine exosomes was examined in a subgroup of the cohort (32 patients with ADPKD and 12 healthy controls). Out of the 44 participants, 21 were males and 23 were females. The cohort was divided into three groups based on participant's

renal function, healthy controls (n = 12), the patients with eGFR > 60 ml/min (n = 16) and the patients with eGFR < 60 ml/min (n = 16).

Table 3.1. Clinical features of the study participants

Characteristic	Healthy controls (Mean ± SEM)	ADPKD patients with eGFR > 60ml/min (Mean ± SEM)	ADPKD patients with eGFR < 60ml/min (Mean ± SEM)	P value
Number	12	26	26	-
Gender (F;M)	7;5	15;11	11;15	0.472
Age (years)	47.42 ± 3.652	44.38 ± 2.36	56.19 ± 2.7	0.0060
BMI (kg/m^2)	28.10 ± 3.714	28.73 ± 5.510	27.48 ± 5.409	0.4175
eGFR (ml/min/1.73m^2)	94.05 ± 22.29	93.65 ± 25.82	34.73 ± 11.57	< 0.0001
eGFR slop (ml/min/1.73m^2)	nm	0.02692 ± 4.933	-4.088 ± 3.407	0.0010
MKL (cm)	nm	13.90 ± 2.422	18.39 ± 4.098	< 0.0001
Cholesterol (mmol/l)	5.488 ±1.002	4.877 ± 0.8887	4.719 ± 0.9078	0.5297
Uric acid (µmol/l)	358.8 ± 92.87	300.2 ± 76.08	386.9 ± 94.97	0.0027
PTH (mmol/l)	nm	35.64 ±17.36	120.3 ± 85.	< 0.0001
Calcium (mmol/l)	2.324 ± 0.022	2.305 ± 0.06941	2.268 ± 0.09123	0.1033
Phosphate (mmol/l)	1.04 ± 0.171	1.058 ± 0.1312	1.109 ± 0.1958	0.0626
PCR (mg/mmol)	nm	12.00 ± 10.32	34.19 ± 65.32	0.0933

The table shows the base line clinical characteristic of the study participants. One-way ANOVA was used to compare the 3 groups and the data presented as mean ± SEM. P values < 0.05 were considered statistically significant and presented in the table with red lines. Dipstick urinalysis of healthy controls was negative for protein, and therefore PCR levels were not measured. Abbreviations are: n; Number of participants, BMI; Body Mass Index, eGFR; estimated Glomerular Filtration Rate, MKL; Mean Kidney Length, PTH; Parathyroid Hormone, PCR; Protein Creatinine Ratio, nm; not measures.

3.7 Analysis of exosomes associated PC1, PC2 and ErbB4 expression in patients with ADPKD and healthy controls

Expression of PC1, PC2, and ErbB4 in urinary exosomes was examined in the patients and healthy controls by western blotting and changes quantified by densitometry of the resulting bands (**Figure 3.4**). For normalization of sample loading, TSG-101 (an exosome specific marker, ~50 kDa) was used as a loading control; this approach has been used in previous studies (Kalani, Mohan et al. 2013; Ho, Yi et al. 2014). All samples were positive for TSG-101 demonstrating successful isolation of the exosome fraction. In these experiments, a high molecular weight band (~400 kDa) (**Figure 3.4A**) was detected using a mouse mAb to PC1 (7e12) specifically raised to the N-terminus of human PC1 (Ong, Harris et al. 1999). As shown, the expression of PC1 was significantly higher in healthy controls compared to the patients (**Figure 3.4B**). A band at ~110 kDa (**Figure 3.4C**) was detected using a PC2 antibody directed against 689–968 amino acids of human PC2 (Santa Cruz Biotechnology, SC-28331). Exosome associated PC2 expression was significantly higher in healthy controls compared to the patients (**Figure 3.4D**).

To my knowledge, ErbB4 expression in urinary exosomes has not been previously described using western blotting. Therefore, the first step was to determine whether exosome associated ErbB4 can be detected in urine by western blotting using a rabbit mAb targeting the C-terminus of ErbB4 (Humtsoe, Pham et al. 2016).This antibody recognised bands at 80 kDa and 50 kDa but not the full length protein (180 kDa) (**Figure 3.4E**). The 80 kDa band most likely represents the intracellular cleaved form of the ErbB4 whereas the 50 kDa band likely represents a proteolytic product of ErbB4. ErbB4 expression in urinary exosomes was significantly higher in patients with ADPKD compared to healthy controls (**Figure 3.4F**).

The expressions of PC1, PC2 and ErbB4 were next examined in a cohort of patients with eGFR > 60 ml/min, patients with eGFR < 60 ml/min and healthy controls by western blotting and the data analysed by one-way ANOVA and the Tukey's post hoc analysis where significant (**Figure 3.5**). For PC1, a decrease in expression as detectable in urine samples of patients with eGFR > 60 ml/min compared to healthy controls (**Figure 3.5A**). However, there was no further decrease in PC1 expression in the patients with eGFR > 60 ml/min when compared to those with eGFR < 60 ml/min (**Figure 3.5B**).

PC2 expression was similarly lower in the 2 patient groups compared to healthy controls (**Figure 3.5C** and **3.5D**). Unlike PC1 however, lower expression of PC2 was observed in patients with eGFR > 60 ml/m group compared to the patients with eGFR

< 60 ml/min. This unexpected result could be explained by the presence of 5 outlier samples in the group of eGFR < 60 ml/m that showed higher expression of PC2 when compared to other participants in this group.

Unlike PC1 and PC2, an 80 kDa C-terminal fragment of ErbB4 was detected mainly in urine samples from the patients with eGFR < 60 ml/min (**Figure 3.5E** and **3.5F**). There was no significant difference in ErbB4 expression between the patients with eGFR > 60 ml/min and healthy controls. A lower band was detected at ~50 kDa which could represent a further cleavage product of ErbB4 or a nonspecific band. However, it was absent in the negative samples and detected with the 80 kDa band suggesting the former possibility.

To gain further insights into the association of exosome associated PC1, PC2 and ErbB4 with the rate of disease progression, their expression was compared in urine samples from ADPKD patients with rapidly progressive disease (eGFR slope > 3 ml/min), ADPKD patients with slowly progressive disease (eGFR slope < 3 ml/min) and healthy controls. One-way ANOVA was used to analyse the data. Overall, significant variations in the expression of the examined proteins were found among the compared groups (**Figure 3.6**). These changes were largely similar to that found when comparing patients separated by baseline eGFR.

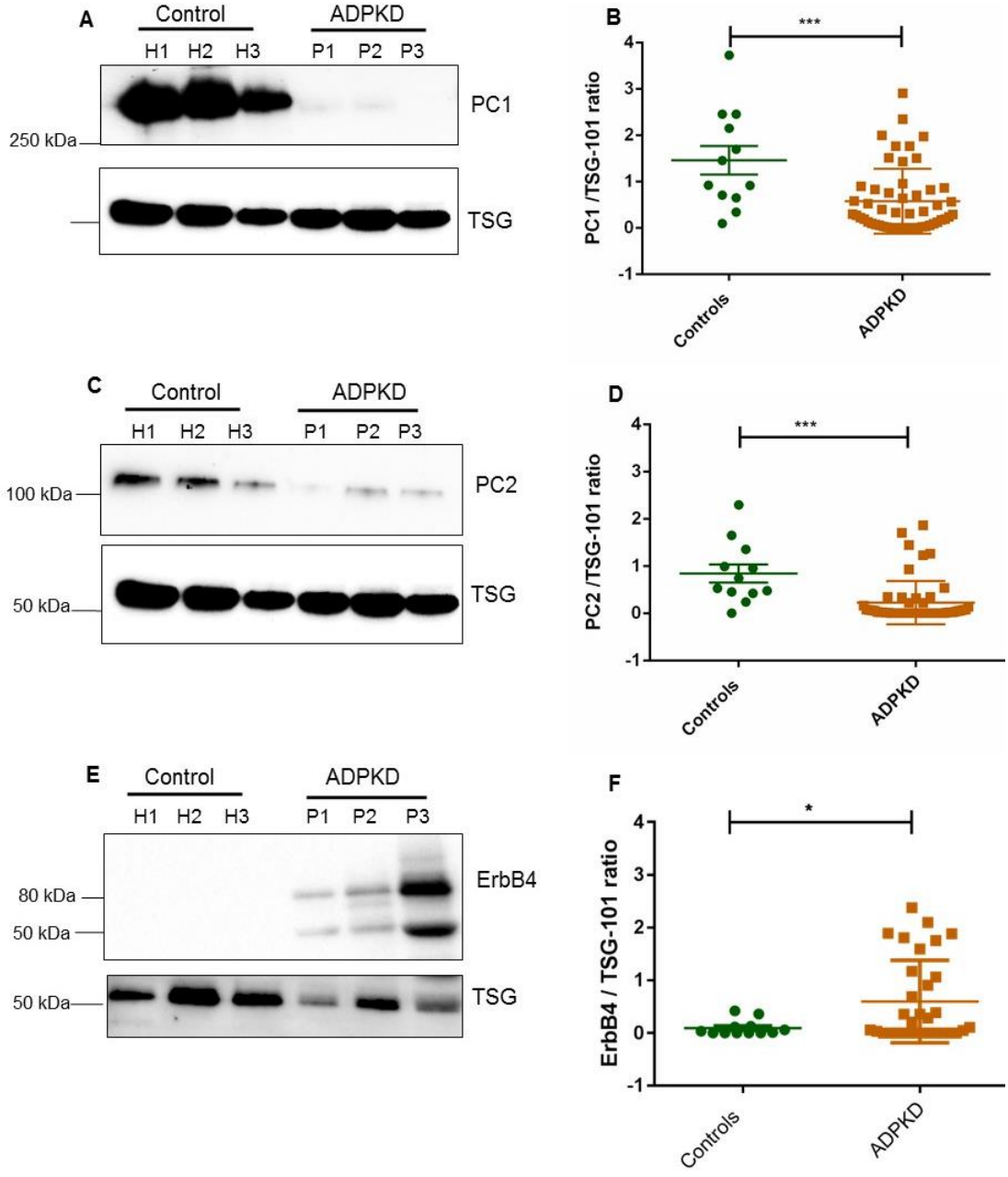

Figure 3.4. Comparison of exosomes associated PC1, PC2 and ErbB4 expression in urine of ADPKD patients and healthy controls

(**A**) A representative western blot for PC1 expression from healthy controls (H1, H2 and H3) and patients with ADPKD (P1, P2, and P3). (**B**) Scatter blotting represents PC1/TSG-101-pixel density quantification in patients with ADPKD (n = 32) compared to healthy controls (n = 12). the averages PC1/TSG-101 expression in urine were 1.462 ± 0.3085 in healthy controls and 0.5719 ± 0.09716 in the patients group, P value = 0.0007.

(**C**) A representative western blot for PC2 expression from healthy controls (H1, H2 and H3) and patients with ADPKD (P1, P2, and P3). (**D**) Scatter blotting represents PC2//TSG-101-pixel density quantification in patients with ADPKD (n = 32) compared to healthy controls (n = 12), the averages PC2/TSG-101 expression in urine were 0.8416 ± 0.1884 in the controls group and 0.2263 ± 0.06350 in the patients group, P value = 0.0003.

(**E**) A representative western blot for ErbB4 expression from healthy controls (H1, H2 and H3) and patients with ADPKD (P1, P2, and P3). (**F**) Scatter blotting represents ErbB4/TSG-101-pixel density quantification in patients with ADPKD (n = 32) compared to healthy controls (n = 12), the averages ErbB4/TSG-101 expression in urine were 0.09289 ± 0.04208 in healthy controls and 0.5952 ± 0.1384 in the patients group, P value = 0.0339.

Independent t test was used to determine the difference between the 2 groups. P value > 0.05 was considered non-significant, * denotes P value < 0.05; ** denotes P value < 0.01; *** denotes P value < 0.001 and **** denotes P value < 0.0001. Data is displayed as mean ± SEM.

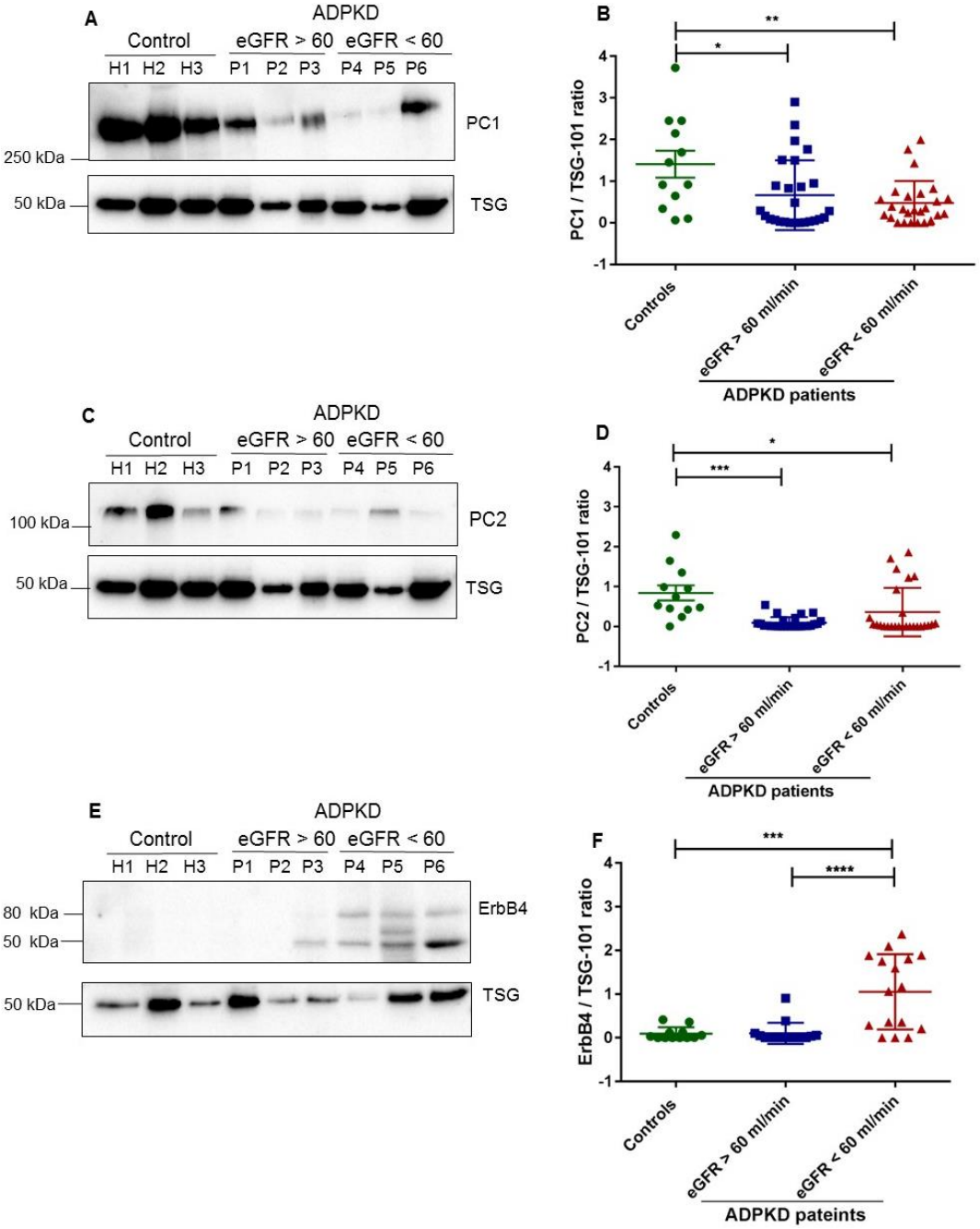

Figure 3.5. Comparison of exosomes associated PC1, PC2 and ErbB4 expression in urine of ADPKD patients with eGFR > 60 ml/min, ADPKD patients with eGFR < 60 ml/min and healthy controls

(**A**) A representative western blot for PC1/TSG-101 expression from healthy controls (H1, H2 and H3), ADPKD patients with eGFR > 60 ml/min (P1, P2, and P3) and ADPKD patients with eGFR < 60 ml/min (P4, P5, and P6). (**B**) Scatter blotting represents PC1/TSG-101-pixel density quantification in urine samples from ADPKD patients with eGFR > 60 ml/min (n = 26), ADPKD patients with eGFR < 60 ml/min (n = 26) and healthy controls (n = 12). The averages (± SEM) of PC1/TSG-101 expression ratio in urine were 1.462 ± 0.3085 in healthy controls, 0.6655 ± 0.1645 in the patients with eGFR > 60 ml/min group and 0.4784 ± 0.1038 in the patients with eGFR < 60 ml/min group, P value = 0.005.

(**C**) A representative western blot for PC2/TSG-101 expression from healthy controls (H1, H2 and H3), ADPKD patients with eGFR > 60 ml/min (P1, P2, and P3) and ADPKD patients with eGFR < 60 ml/min (P4, P5, and P6). (**B**) Scatter blotting represents PC2/TSG-101-pixel density quantification in urine samples from ADPKD patients with eGFR > 60 ml/min (n = 26), ADPKD patients with eGFR < 60 ml/min (n = 26) and healthy controls (n = 12). The averages (± SEM) of PC2/TSG-101 expression ratio in urine were 0.8416 ± 0.1884 in healthy controls, 0.09359 ± 0.02805 in the patients with eGFR > 60 ml/min group and 0.3588 ± 0.1194 in the patients with eGFR < 60 ml/min group, P value = 0.0002.
(**E**) A representative western blot for ErbB4/TSG-101 expression in urine samples from healthy controls (H1, H2 and H3), ADPKD patients with eGFR > 60 ml/min (P1, P2, and P3) and ADPKD patients with eGFR < 60 ml/min (P4, P5, and P6). (**F**) Scatter blotting represents ErbB4/TSG-101-pixel density quantification in ADPKD patients with eGFR > 60 ml/min (n = 16), ADPKD patients with eGFR < 60 ml/min (n = 16) and healthy controls (n = 12). The averages (± SEM) of ErbB41/TSG-101 expression ratio in urine were 0.09289 ± 0.04208 in healthy controls, 0.1022 ± 0.06278 in the patients with eGFR > 60 ml/min group and 1.030 ± 0.2039 in the patients with eGFR < 60 ml/min group, P value < 0.0001.

One-way ANOVA followed by a Tukey's post hoc analysis was used to determine the differences between the 3 groups. P value > 0.05 was considered non-significant, * denotes P value < 0.05; ** denotes P value < 0.01; *** denotes P value < 0.001 and **** denotes P value < 0.0001. Data is displayed as mean ± SEM.

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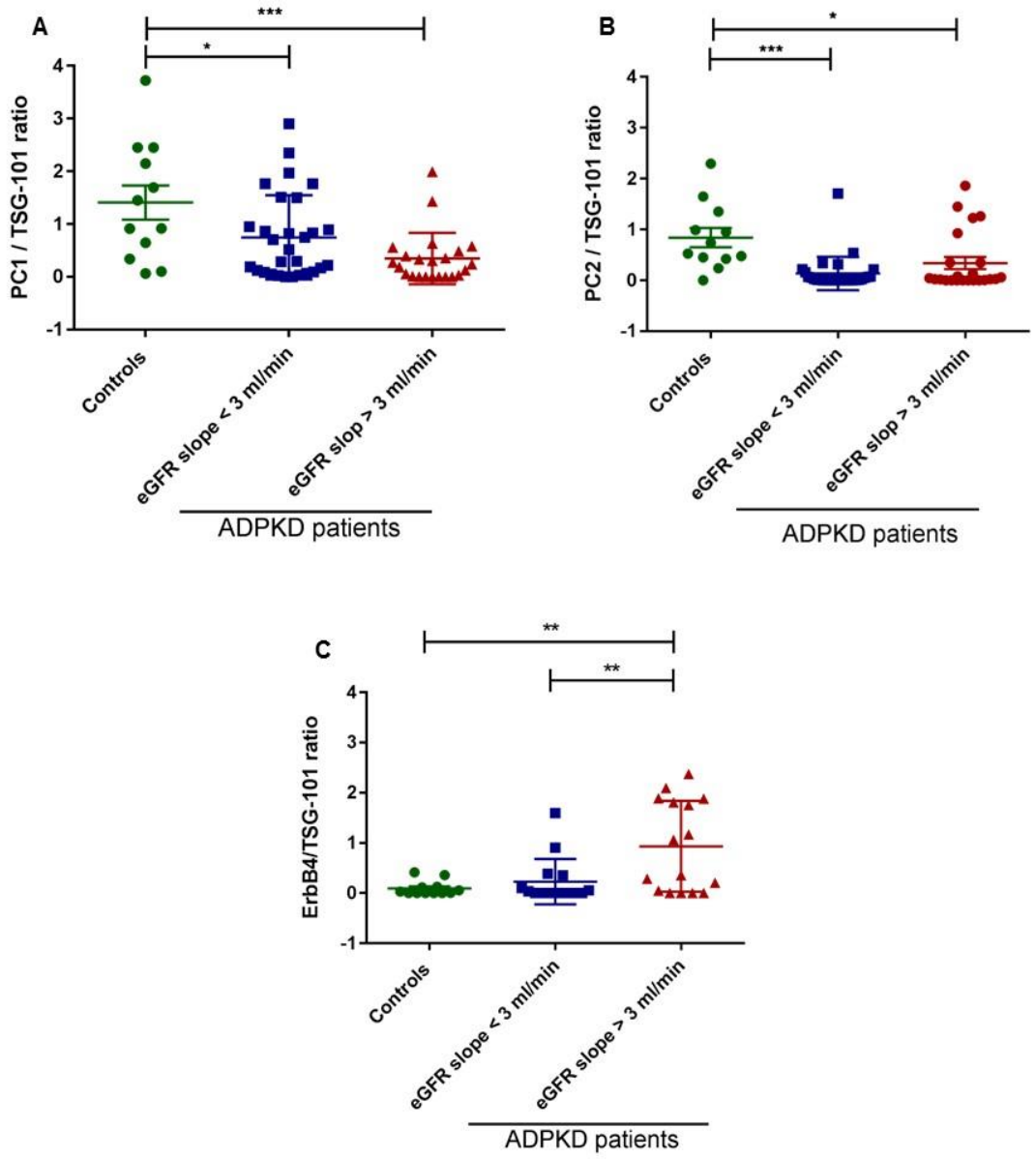

Figure 3.6. Comparison of exosomes associated PC1, PC2 and ErbB4 expression in urine of ADPKD patients with eGFR slope < 3 ml/min, ADPKD patients with eGFR slope > 3 ml/min and healthy controls

(**A**) A scatter blotting represents comparisons of PC1/TSG-101 expression in urine samples from healthy controls (n = 12), the patients with eGFR slope < 3 ml/min over 5 years (n = 29) and ADPKD patients with eGFR slope > 3 ml/min over 5 years (n = 23). The averages (± SEM) of PC1/TSG-101 expression ratio in urine were 1.409 ± 0.3243 in healthy controls, 0.7498 ± 0.1479 in the patients with eGFR slope < 3 ml/min group and 0.3477 ± 0.1014 in the patients with eGFR slope > 3 ml/min group, P value = 0.0014.

(B) A scatter blotting represents comparisons of PC2/TSG-101 expression in urine samples from healthy controls (n = 12), the patients with eGFR slope < 3 ml/min over 5 years (n = 29) and ADPKD patients with eGFR slope > 3 ml/min 5 years (n = 23). The averages (± SEM) of PC2/TSG-101 expression ratio were 0.8416 ± 0.1884 in healthy controls, 0.1349 ± 0.06110 in

the patients with eGFR slope < 3 ml/min group and 0.3413 ± 0.1186 in the patients with eGFR slope > 3 ml/min group, P value = 0.0005.

(**C**) A scatter blotting represents comparisons of ErbB4/TSG-101 expression in urine samples from healthy controls (n = 12), the patients with eGFR slope < 3 ml/min over 5 years (n = 16) and ADPKD patients with eGFR slope > 3 ml/min over 5 years (n = 16). The averages (± SEM) of ErbB4/TSG-101 expression ratio in urine were 0.09289 ± 0.04208 in healthy controls, 0.2290 ± 0.1167 in the patients with eGFR slope < 3 ml/min group and 0 .9183 ± 0.2131 in the patients with eGFR slope > 3 ml/min group, P value = 0.0012.

One-way ANOVA followed by a Tukey's post hoc analysis was used to determine the differences between the 3 groups. P value > 0.05 was considered non-significant, * denotes P value < 0.05; ** denotes P value < 0.01; *** denotes P value < 0.001 and **** denotes P value < 0.0001. Data is displayed as mean ± SEM.

3.8 Association of exosomes associated PC1, PC2 and ErbB4 expression in urine with conventional measures of ADPKD

Next, PC1, PC2 and ErbB4 expression in urine exosomes was correlated with other measures of kidney disease i.e. eGFR and MKL. The association between candidate proteins with eGFR and MKL is depicted in **Figure 3.7** and **Figure 3.8**. ErbB4 showed the strongest correlation with eGFR followed by PC2. There was no correlation however between PC1 expression and eGFR. None of the three proteins showed a significant correlation with MKL.

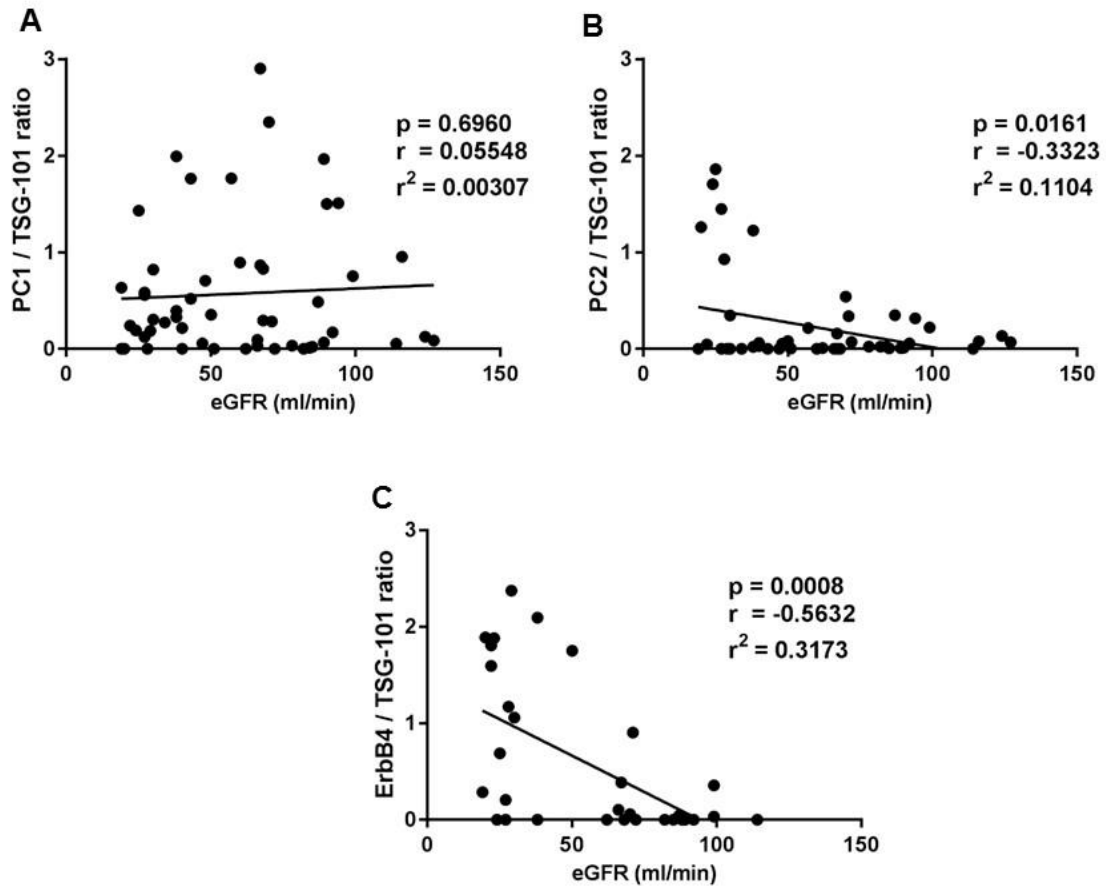

Figure 3.7. Association of exosomes associated PC1, PC2 and ErbB4 expression in urine with eGFR

The figure shows the correlation between exosomes associated (**A**) PC1/TSG-101 ratio, (**B**) PC2/TSG-101 ratio and (**C**) ErbB4/TSG-101 ratio with eGFR in patients with ADPKD. Spearman logistic regression revealed significant associations between PC2/TSG ratio and ErbB4/TSG ratio with eGFR.

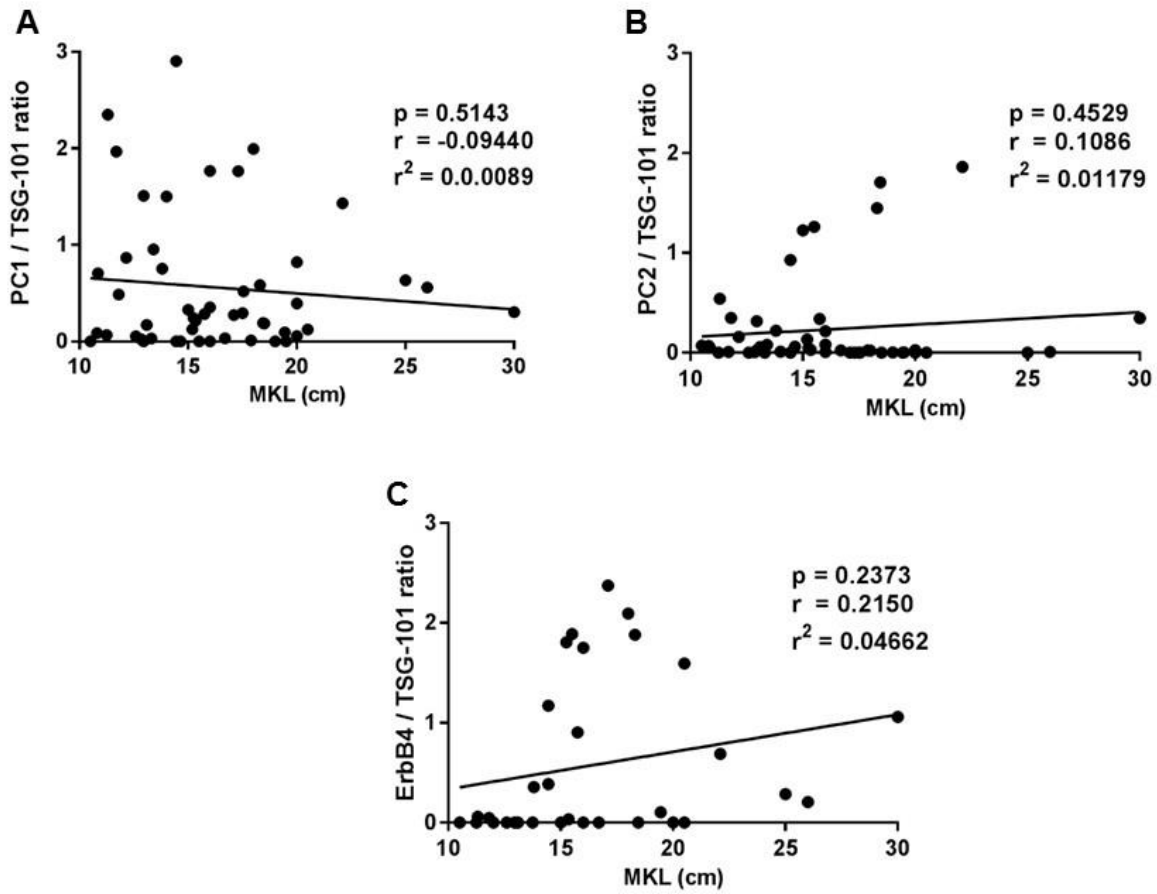

Figure 3.8. Association of exosomes associated PC1, PC2 and ErbB4 expression in urine with MKL

The figure shows the correlation between exosomes associated (**A**) PC1/TSG-101 ratio, (**B**) PC2/TSG-101 ratio and (**C**) ErbB4/TSG-101 ratio with MKL in patients with ADPKD. Spearman logistic regression revealed no significant associations between the examined proteins and MKL.

3.9 Univariate and multivariate regression analysis

A linear regression analysis was carried out to identify the correlations between the examined exosomes associated proteins and other clinical variables (**Table 3.2**). No statistically significant univariate correlations were found between the examined proteins and the mean age, BMI, MKL, cholesterol as well as serum uric acid. Only eGFR slope showed a significant association with PC1, whereas, both eGFR and protein creatinine ratio showed significant associations with PC2. Estimated GFR, eGFR slope, calcium, protein creatinine ratio and PTH showed significant univariate correlations with ErbB4 expression.

All the clinical and biochemical parameters of the patients with ADPKD that showed a significance univariate association with the examined proteins were included in a multiple regression analysis model (**Table 3.3**). Estimated GFR slope remained significantly associated with PC1, whereas, only PCR showed a significant association with PC2. Estimated GFR, eGFR slope, PCR and calcium lost their significant association with ErbB4 expression in this analysis. Only PTH remained significantly associated with ErbB4 expression.

Table 3.2. Univariate linear regression of exosomes associated PC1, PC2 and ErbB4 expression in urine with clinical and biochemistry variables of ADPKD patients

Variables	PC1/ TSG-101 ratio Standardized β (p value)	PC2/ TSG-101 ratio Standardized β (p value)	ErbB4/ TSG-101 ratio Standardized β (p value)
Age (year)	0.162 (0.250)	0.226 (0.110)	0.137 (0.373)
BMI (kg/m^2)	-0.061 (0.670)	-0.154 (0.285)	-0.306 (0.058)
eGFR (ml/min/1.73m^2)	0.030 (0.834)	-0.314 (0.025)	-0.579 (0.001)
eGFR slope (ml/min/1.73m^2)	0.298 (0.032)	-0.080 (0.579)	-0.414 (0.018)
MKL (cm)	-0.094 (0.514)	0.112 (0.445)	0.238 (0.189)
Cholesterol (mmol/l)	0.153 (0.279)	0.192 (0.176)	-0.175 (0.280)
Calcium (mmol/l)	0.094 (-0.508)	-0.071 (0.622)	-0.355 (0.046)
Uric acid (µmol/l)	-0.166 (0.238)	0.166 (0.245)	0.2333 (0.149)
PCR (mg/mmol)	-0.073 (0.634)	0.497 (0.001)	0.392 (0.027)
PTH (mmol/l)	-0.247 (0.102)	0.294 (0.053)	0.640 (0.000)

A linear regression analysis for the various clinical and biochemical variables was generated to examine the independent associations between these parameters and PC1/TSG-101 ratio, PC2/TSG-101 ratio and ErbB4/TSG-101 ratio. Standardized β (standardized coefficients beta) denotes that an alteration of 1SD in the clinical parameters (independent variable) will lead to a one measurement change in the corresponding protein (dependent factor). P value < 0.05 was considered statistically significant and denotes with red lines. Abbreviations are: BMI; Body Mass Index, eGFR; estimated Glomerular Filtration Rate, MKL; Mean Kidney Length, PTH; Parathyroid Hormone, PCR; Protein Creatinine Ratio.

Table 3.3. Multivariate linear regression of exosomes associated PC1, PC2 and ErbB4 expression in urine with clinical and biochemistry variables of ADPKD patients

Variables	PC1/TSG-101 ratio Standardized β (p value)	PC2/TSG-101 ratio Standardized β (p value)	ErbB4/TSG-101 ratio Standardized β (p value)
eGFR (ml/min/1.73m^2)	/	/	-0.127 (0.528)
eGFR slope (ml/min/1.73m^2)	0.298 (0.032)	0.101 (0.480)	-0.021 (0.901)
Calcium (mmol/)	/	/	0.455 (0.124)
PTH (mmol/)	/	/	-0.455 (0.038)
PCR (mg/mmol)	/	0.466 (0.002)	-0.218 (0.146)

The table shows a multivariate regression model adjusted to the variables which have significant correlations with the corresponding protein.

3.10 Prediction of ADPKD disease progression

Finally, the ability of exosomes associated PC1/TSG-101 ratio, PC2/TSG-101 ratio and ErbB4/TSG-101 ratio to predict ADPKD progression was calculated based on eGFR slope using ROC curves (SPSS, version 22). Estimated GFR slope was measured based on the decline in renal function over the past 5 years. A loss of renal function > 3 ml/min/1.73m^2 was considered as rapidly progressive disease (Rosansky and Glassock 2014). A ROC curve calculation relies on the fraction of the number of true positives (study sensitivity) and the number of false positives (1 - sensitivity).

The prediction ability of disease progression based on the PC1/TSG-101 ratio or PC2/TSG-101 ratio or a combination of the 2 proteins was not better than by chance (**Figure 3.9A**) whereas ErbB4/TSG-101 ratio had a significant ability to predict ADPKD progression which outperformed the ability of MKL (measured by US) (**Figure 3.9B** and **C**). A combination of ErbB4/TSG-101 and MKL improved the prediction ability further (**Figure3.9D**). The exact AUC, 95% CI and the P values of each protein, MKL, proteins combination and combination of proteins and MKL in predicting disease progression are displayed in **Table 3.4**. Under these experimental conditions, the cut-off value of ErbB4/TSG-101 ratio that might be helpful in distinguishing between patients with slowly progressive disease from those with rapidly progressive disease was 0.154 (70.6% sensitivity and 73.3% specificity), whereas, a cut-off value of mean kidney length of 15.1 ml provided 70.6% sensitivity and 53.3% specificity.

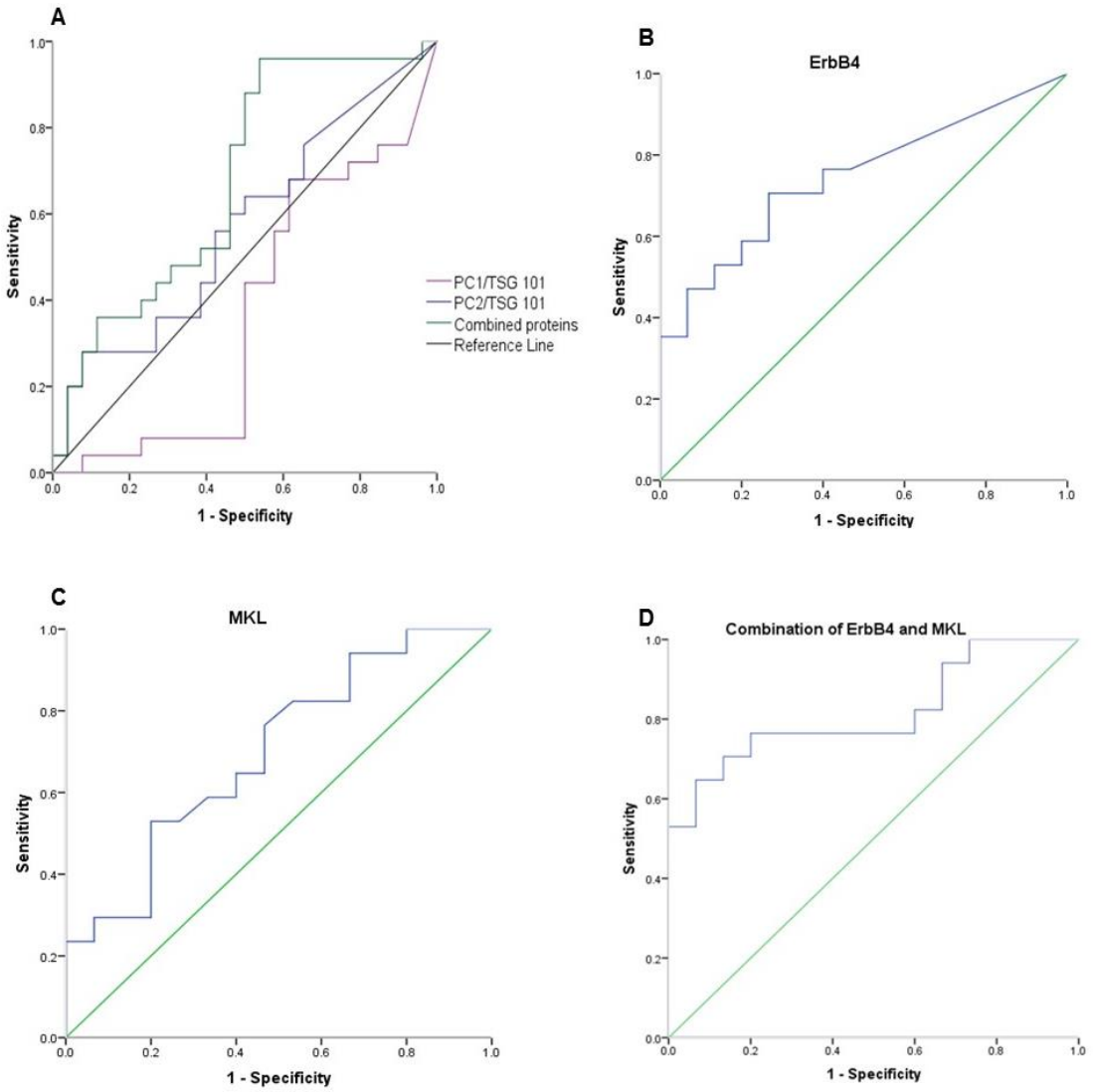

Figure 3.9. Diagnostic performance of exosomes associated PC1, PC2 and ErbB4 expression in urine to predict ADPKD progression

ROC curves for exosome associated proteins for dichotomized eGFR slope. In comparison to the MKL, ErbB4 showed a higher AUC with a significant P value which reflecting a better ability to discriminate between patients with the risk of rapidly progressive disease from the patients with slowly progressive disease risk. Abbreviations are: ROC curve; Receiver operating Curve, MKL; Mean Kidney Length. AUC and P value of each protein and in combination with other proteins and MKL are shown in the table 3.5.

Table 3.4. Diagnostic performance of exosomes associated PC1, PC2 and ErbB4 expression in urine to predict ADPKD progression

Variables	AUC	95% CI	P value
PC1/TSG-101 ratio	0.393	0.230-0.557	0.083
PC2/TSG-101 ratio	0.545	0.382-0.708	0.589
ErbB4/TSG-101 ratio	0.745	0.573-0.917	0.018
Mean kidney length (MKL)	0.698	0.516-0.88	0.057
Combination of PC1/TSG-101 ratio and PC2/TSG-101 ratio	0.663	0.509-0.818	0.05
Combination of MKL and ErbB4/TSG-101 ratio	0.816	0.665-0.967	0.002

ROC curves for PC1, PC2, ErbB4 and MKL for dichotomized eGFR slope to predict ADPKD progression. Protein combination with MKL showed the highest AUC which reflecting the ability to discriminate between patients with risk of CKD rapidly progression from patients with slowly progressive risk. Abbreviations are: ROC curve; Receiver operating Curve, CI; Confidence Interval, P value; probability value

3.11 Summary

The key findings in this chapter were:

1. Expression of PC1 and PC2 in urine exosomes was significantly reduced in patients with ADPKD compared to healthy controls.

2. Exosomal ErbB4 was detectable as a C-terminal 80 kDa cleavage product with a lower 50 kDa band of uncertain significance.

3. Expression of ErbB4 was significantly increased in patients with ADPKD with reduced renal function (eGFR < 60) when compared to healthy controls or patients with preserved eGFR (> 60).

4. Exosome PC2 and ErbB4 expression were significantly correlated with baseline eGFR.

5. Exosome ErBb4 expression was the best predictor of ADPKD progression (eGFR slope over 5 years) compared to PC1, PC2 and MKL (measured by US) by ROC analysis.

3.12 Discussion

Urinary exosomes can be excreted by epithelial cells from different nephron segments (Edelstein 2011; Yuana, Sturk et al. 2013). The protein components of exosomes reflect their cell of origin and are typically enriched with over 100 proteins including cytoplasmic and membrane proteins (Pisitkun, Shen et al. 2004; Gonzales, Pisitkun et al. 2009; van Balkom, Pisitkun et al. 2011). In addition, exosomes may protect cargo molecules from degradation by extracellular enzymes (Buzas, Gyorgy et al. 2014). Recently, there has been a growing interest in urine exosome associated proteins as potential diagnostic biomarkers for kidney diseases. Increased expression of urine exosome associated Wilms' tumour-1 protein has been found to predict a risk of renal function deterioration in patients with type-1 diabetes mellitus (Kalani, Mohan et al. 2013). Additionally, Zhou et al. reported a 31-fold increase in urine exosome associated fetuin-A expression following acute nephrotoxic exposure which preceded an elevation in serum creatinine (Zhou, Pisitkun et al. 2006).

In this project, I confirmed that exosomes are released into urine from healthy volunteers and ADPKD patients. The isolated vesicles were consistent with the typical reported characteristics of exosomes as confirmed by examination of their morphological and biochemical characteristics. Their morphological features by electron microscopy revealed small round vesicles with a diameter of 40-100 nm consistent with previous reports (Thery, Boussac et al. 2001; van Niel, Raposo et al. 2001; Pisitkun, Shen et al. 2004; Kesimer, Scull et al. 2009). Biochemically, there was an enrichment of the exosome marker TSG-101 (Alvarez, Khosroheidari et al. 2012). TSG-101 is a component of endosomal sorting complex required for transport (ESCRT) complex which is implicated in the trafficking of endosomal cargo to the MVBs and further exosomes formation (Hurley 2008; Horgan, Hanscom et al. 2012). Inhibition of ESCRT or depletion of TSG-101 leads to a decrease in exosome secretion from cancer cells (Colombo, Moita et al. 2013; Ha, Yang et al. 2016). The presence of AQP-2, a marker of renal collecting duct, in urine exosomes indicated its origin from principal cells of the collecting duct (Keller, Rupp et al. 2007; Yuana, Sturk et al. 2013; Salih, Demmers et al. 2016). AQP-2 is the main water channel regulated by vasopressin to regulate water reabsorption by the collecting ducts (Nielsen, Chou et al. 1995; Kwon, Frokiaer et al. 2013).

Mutations in *PKD1* or *PKD2* account for almost all patients with ADPKD. They encode for the PC1 and PC2 proteins which in previous studies, have been shown to be excreted in urine exosomes (Pisitkun, Shen et al. 2004; Hogan, Manganelli et al.

2009). I hypothesized that changes in urinary excretion of exosome associated PC1 or PC2 could act as early diagnostic or prognostic markers in ADPKD.

The major finding of this study was the downregulation of normalised PC1 and PC2 in ADPKD urine exosomes compared to healthy controls. During the course of this project, Pocsfalvi et al. reported that PC1 and PC2 were absent in urine samples of ADPKD patients compared to healthy controls using ITRAQ labelling mass spectrometry and western blotting (Pocsfalvi, Raj et al. 2015). In contrast, Hogan et al. reported a reduction (but not absence) in PC1 and PC2 expression in ADPKD urine exosomes compared to healthy controls by MS based proteomics analysis (Hogan, Bakeberg et al. 2015). My results confirm the latter results and suggest that urine exosome PC1 or PC2 could be used as a diagnostic marker for ADPKD.

To examine the utility of exosome PC1 and PC2 as a prognostic marker, their expression was correlated with baseline kidney function (eGFR), mean kidney length (as a surrogate marker of disease) and the rate of disease progression (eGFR slope over 5 years). In multivariate analysis, PC1 expression showed significant correlations with the eGFR slope but not baseline eGFR (**Table 3.3**). A likely explanation is that patients with truncating *PKD1* mutations (who also have the most rapid disease progression) are likely not to produce PC1 from the mutant allele and therefore will have less than 50% PC1 expression compared to those with *PKD1* non-truncating mutations (Cornec-Le Gall, Audrezet et al. 2013; Ong, Devuyst et al. 2015; Hwang, Conklin et al. 2016).

An important finding of this study was of increased expression of the 80 kDa C-terminal intracellular domain of ErbB4 in urine exosomes from ADPKD patients with eGFR < 60 ml/min when compared to the patients with eGFR > 60 ml/min and healthy controls. Upon activation of ErbB4 by EGF related growth factors, mainly neuregulin 1, ErbB4 is cleaved to an ectodomain fragment (120 kDa) and a membrane associated domain (80 kDa) by tumour necrosis factor alpha converting enzyme (TACE) (Vecchi, Baulida et al. 1996; Cheng, Tikhomirov et al. 2003) (**Figure 3.10**). The extracellular domain (approximately 120 kDa) is released into the extracellular space, explaining its absence from the exosomes seen in this study (Vecchi, Baulida et al. 1996). The membrane associated intracellular fragment is further processed by γ-secretase within the transmembrane domain to generate a soluble intracellular domain which translocate to the nucleus mediating gene transcription (Ni, Murphy et al. 2001; Lee, Jung et al. 2002; Linggi, Cheng et al. 2006). The expression of this isoform in late stage disease implicates its role in disease progression.

In univariate analysis, exosome ErbB4 expression showed significant correlations with baseline eGFR and eGFR slope though this was not conserved in multivariate analysis. A surprising finding was a correlation between PTH levels and ErbB4 expression (**Table 3.3**). Previous studies had reported that PTH stimulates cleavage of HB-EGF and further activates EGF signals in distal renal tubules (HEK-293 cells) (Sneddon, Yang et al. 2007). However, this has not been shown for ErbB4. In osteoblast, PTH increases the expression of EGFR which is mediated by an increase in cAMP production (Drake, Baldassare et al. 1994; Gonzalez, Disthabanchong et al. 2002). Additionally, activation of the EGFR family is requisite to mediate activation of mitogenesis in bone cells by PTH (Fang, Kujubu et al. 1992; Ahmed, Gesty-Palmer et al. 2003). Further work is required to examine whether PTH is itself causally relevant to high expression of ErBb4 or an association relationship with the progressive of renal impairment.

By ROC analysis, ErbB4 was the best marker for disease progression (eGFR slope) outperforming MKL (**Table 3.4**). The combination of ErbB4 and MKL further improved AUC to 0.816.

Our ErbB4 results validate in vitro studies by our laboratory which showed over expression of ErbB4 in different ADPKD models compared to healthy controls using microarray, qPCR, western blotting and immunohistochemistry, and suggests a significant role for ErbB4 in the pathogenesis of ADPKD (Streets, Magayr et al. 2017). Consistently, abnormal expression of EGFR, EGF, ErbB4 and HB-EGF has been observed in cystic kidney of bpk mouse model of ADPKD compared to normal kidney of a wild type (Nemo, Murcia et al. 2005). EGF-ErbB receptors, in particular heparin-binding EGF, play a key role in cell proliferation during renal development as well as during cell regeneration, in addition, it stimulates cyst formation and growth (MacRae Dell, Nemo et al. 2004; Jiang, Chiou et al. 2006; Zheleznova, Wilson et al. 2011; Coaxum, Blanton et al. 2014). Intriguingly, heparin-binding EGF-like growth factor (HB-EGF) activates tubular formation in Madin-Darby canine kidney cell through induce phosphorylation and nuclear translocation of 80 kDa cytoplasmic cleavage of JM-a/CYT-2 cells (Zeng, Zhang et al. 2007). What is important in this stimulation is identification of HB-EGF as a predictor marker of ADPKD progression (Harskamp, Gansevoort et al. 2015). Harskamp et al. examined the expression of HB-EGF in plasma and urine samples from 27 patients with ADPKD and 27 healthy controls. The authors found a higher excretion of HB-EGF in urine and serum samples from ADPKD patients when compared to healthy controls, and found a positive correlation in its expression with the severity of ADPKD.

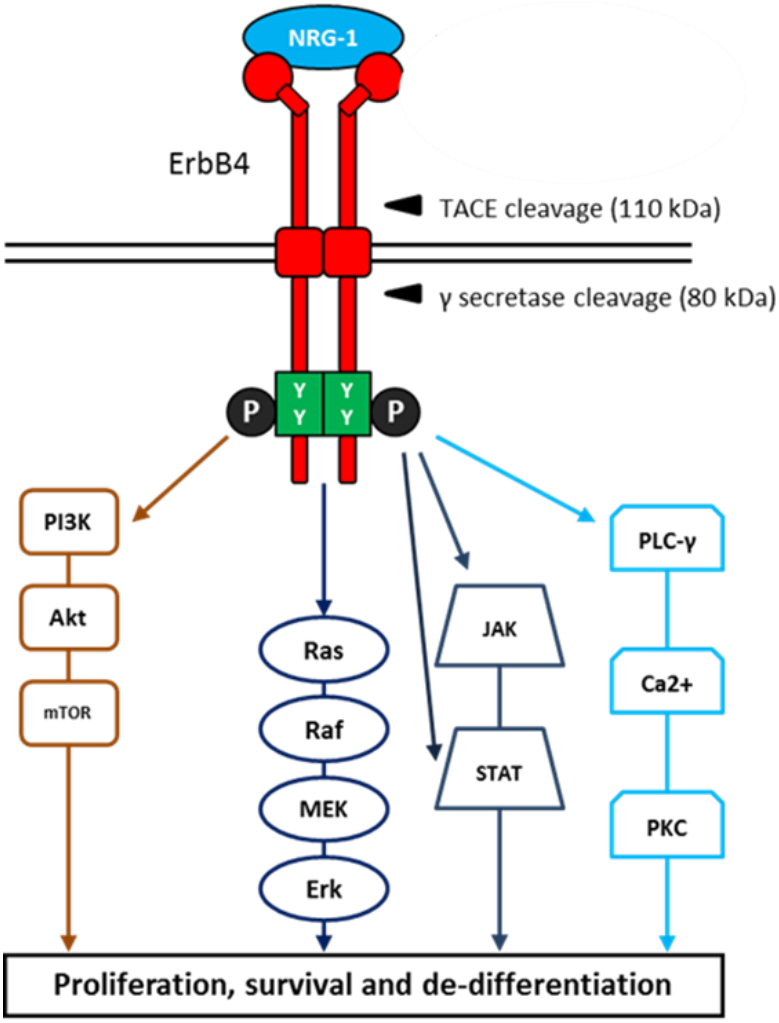

Figure 3.10. Cleavage of the ErbB4 ectodomain

ErbB4 (180 kDa) composes of extracellular ligand binding region, an intracellular cytoplasmic domain and a single transmembrane region, and it is activated by neuregulin 1-4 ligands (Elenius, Choi et al. 1999, Veikkolainen, Vaparanta et al. 2011). Binding of NRG-1 to ErbB4R induces its cleavage by TACE followed by γ-secretes to generate S80 intracellular domain. Generation of 80 kDa fragment leads to signal cascade activations to stimulate proliferation, survival and de-differentiation of the renal epithelial cells (Streets, Magayr et al. 2017).

Chapter 4 Urinary microRNAs as biomarkers for disease progression

4.1 Introduction

Since their discovery in 1993 (Lee, Feinbaum et al. 1993), microRNAs have attracted much attention as potential diagnostic or prognostic biomarkers in many field including cancer, cardiac, renal and autoimmune diseases (Wang, Li et al. 2011; Yin, Li et al. 2012; Zeng, Cui et al. 2014; Zhou, Gong et al. 2015). In the field of kidney disease, microRNAs have been suggested to play significant roles in the initiation and progression of different forms of kidney diseases (Amrouche, Bonifay et al. 2011; Neal, Michael et al. 2011; Lorenzen and Thum 2012; Trionfini, Benigni et al. 2015). These include acute kidney injury (Aguado-Fraile, Ramos et al. 2015); systemic lupus nephritis (Wang, Tam et al. 2010); IgA nephropathy (Wang, Kwan et al. 2011); chronic renal impairments (Szeto, Ching-Ha et al. 2012); idiopathic nephrotic syndrome (Luo, Wang et al. 2013); and diabetic nephropathy (Rezk, Sabbah et al. 2016).

A fundamental requirement for biomarker research in clinical applications is stability under various conditions. Significantly, microRNAs are stable under various conditions including multiple freeze/thaw cycles, boiling and pH variability (Chen, Ba et al. 2008; Turchinovich, Samatov et al. 2013). In contrast, other RNA species, including rRNA, tRNA and mRNA are susceptible to nuclease mediated degradation in the extracellular environment (Chen, Ba et al. 2008; Turchinovich, Weiz et al. 2011; Turchinovich, Samatov et al. 2013). Additionally, the long term stability of urine exosomal microRNAs when stored at -80^{o}C (up to 12 months) has been confirmed (Lv, Cao et al. 2013). It is notable that urinary microRNAs are likely to originate from renal tubular epithelial cells instead of overflow from blood circulation and can therefore provide non-invasive information about kidney structure and function (Wang, Tam et al. 2010; Yun, Jeong et al. 2012).

A limited number of studies have examined the direct pathogenic role of individual microRNAs in ADPKD (**Table 1.7**). Alteration of microRNA expression has been reported in urine, cystic lines and animal models of ADPKD (Sun, Li et al. 2010; Dweep, Sticht et al. 2013; Patel, Williams et al. 2013; Ben-Dov, Tan et al. 2014). In one study, down regulation of a miR-200 family in Dicer knockout mice or inhibition of miR-200 in renal epithelial cells was associated with increased *PKD1* mRNA expression and cystogenesis (Patel, Hajarnis et al. 2012). Since microRNAs can directly alter expression of *PKD1* and *PKD2* as well as influence other disease processes, it seems likely that the differential expression of specific microRNAs can alter disease severity and therefore represent prognostic biomarkers of disease.

4.2 Hypothesis

Urinary microRNAs may be prognostic biomarkers of renal outcome in ADPKD. This hypothesis was examined by investigating the differential expression of extracellular microRNAs in cell free and exosomal fractions of urine between patients and matched healthy controls.

4.3 Aim

The key aim of this chapter was to identify differentially expressed urinary microRNAs which could act as potential early biomarkers of rapid disease progression in ADPKD.

4.4 Objectives

To achieve this aim, the following objectives were performed:

1. Identification of deregulated microRNAs in retrospective cell free urine samples from patients with ADPKD compared to healthy controls by qPCR based microRNA arrays.

2. Identification of differential expression exosomal associated microRNAs by next generation sequencing.

3. Validation and quantification of the differential expressed microRNAs in urine samples from patients with various stages ADPKD (eGFR > or < 60 ml/min/1.73m^2) and healthy controls.

4. Correlate the differentially expressed microRNAs with structural or functional measures of renal function including eGFR, eGFR slope and mean kidney length (MKL).

5. Calculate the potential ability of the differentially expressed microRNAs to predict ADPKD progression.

6. Identify enriched pathways and potential target genes of the differentially expressed microRNAs using bioinformatics.

7. Examine the expression of several potential differentially expressed genes in urine exosomes from patients and healthy controls.

4.5 Identification of deregulated microRNAs in cell free urine

MicroRNAs have been detected in different urine fractions including cell free urine (Yun, Jeong et al. 2012). The precise mechanism of extracellular microRNA release into biofluids is still unknown (Yun, Jeong et al. 2012). Beside the export of microRNAs within microvesicles from cells, a fraction of microRNAs are released from cells and protected from degradation by forming a complex with Argonaut proteins (Arroyo, Chevillet et al. 2011; Turchinovich, Weiz et al. 2011).

Previous studies demonstrated that cell free microRNAs exist stably in various biofluids including urine and correlate with disease activity (Mitchell, Parkin et al. 2008; Wang, Zhang et al. 2009; Wang, Tam et al. 2010; Wang, Tam et al. 2011). In addition, isolation of microRNAs from cell free urine is a simple method that can be easily applied to clinical situations. Therefore, a pilot project was initiated to investigate if isolation of microRNAs from cell free urine could reliably predict disease progression in ADPKD. **Figure 4.1** shows the workflow of these experiments.

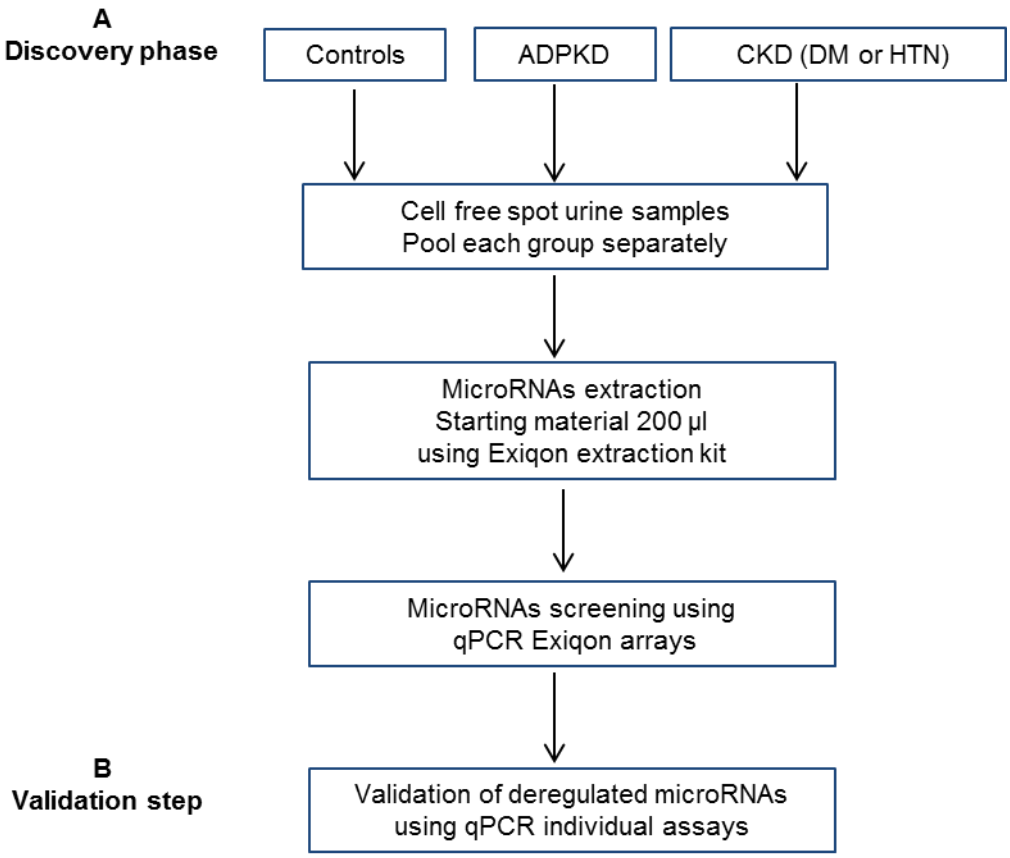

Figure 4.1. Flow chart for cell free associated microRNAs analysis

The graph shows the flow chart of microRNAs discovery phase in cell free urine. Screening of 752 microRNAs were performed using Exiqon panel 1 and 2. Abbreviations are: eGFR; estimated Glomerular Filtration Rate, ADPKD; Autosomal Dominant Polycystic Kidney Disease, CKD; Chronic Kidney Disease, DM; Diabetic Mellitus, HTN; Hypertension.

4.5.1 Baseline characteristics of the study participants

Retrospectively collected urine samples were obtained from the Sheffield Kidney Institution (SKI) bio-repository. These samples were routinely collected from nephrology outpatients as a longitudinal biomarker study into CKD pathogenesis (2009-2010). Out of 400 urine specimens, 20 samples were selected to be examined in this pilot study; 5 samples from each group were pooled together (5 ADPKD patients, 5 diabetic patients, 5 hypertension patients and 5 healthy controls). The clinical features of the selected participants are summarized in the **Table 4.1**. There was significant difference in the mean age between the examined groups, diabetic patients were older than ADPKD patients, whereas, no significant differences between the hypertension group and ADPKD or diabetic groups. No statistically differences between the groups regarding gender, eGFR and PCR.

4.5.2 MicroRNA discovery qPCR array in retrospective samples

Quantitative PCR arrays were carried out to identify distinctive cell free microRNAs on pooled samples of patients with ADPKD, DM, HTN and healthy controls (**Section 2.7**) using Exiqon panel 1 and 2. 3 differentially expressed microRNAs were detected only in samples from the patients with CKD (DM or HTN), and only 2 differentially expressed microRNAs detected in the samples from the control group. 7 differentially expressed microRNAs were identified differentially expressed in pooled urine samples from patients with ADPKD (with CT values > 35) when compared to the other examined groups, including miR-22-5p, miR-24-3p, miR-93-5p, miR-99b-5p, miR-151a-5p, miR-205-5p and miR-429 (**Figure 4.2**).

I next validated these differentially expressed microRNAs in individual samples using qPCR. However, the results of ADPKD specific microRNAs showed no significant differences between ADPKD and controls with high CT values (reflecting low abundance) for all samples (**Figure 4.3 A** to **H**).

Table 4.1. Clinical features of the study participants

Variable	Control	ADPKD	DM	HTN	P value
Age (years)	/	51.40 ± 7.922	75.60 ± 3.415	71.60 ± 6.501	0.0385
Gender (F;M)	2;3	2;3	2;3	2;3	1
eGFR (ml/min/1.73 m^2)	nm	39.40 ± 12.98	29.80 ± 4.236	24.20 ± 2.835	0.4281
PCR (mg/mmol)	/	21.60 ± 4.273	39.60 ± 7.026	78.50 ± 62.50	0.4577

The table shows the basic demographic and clinical features of the participants in the microRNAs discovery phase. The data was analysed using one- way ANOVA test, a Tukey's post hoc analysis was used to find the source of insignificant. The table data expressed as mean ± SEM. Abbreviations are: Autosomal Dominant Polycystic Kidney Disease, CKD; Chronic Kidney disease, DM; Diabetic Mellitus, HTN; Hypertension, F; Female, M; Male, eGFR; estimated Glomerular Filtration Rate, PCR; Protein Creatinine Ratio, nm; not measured.

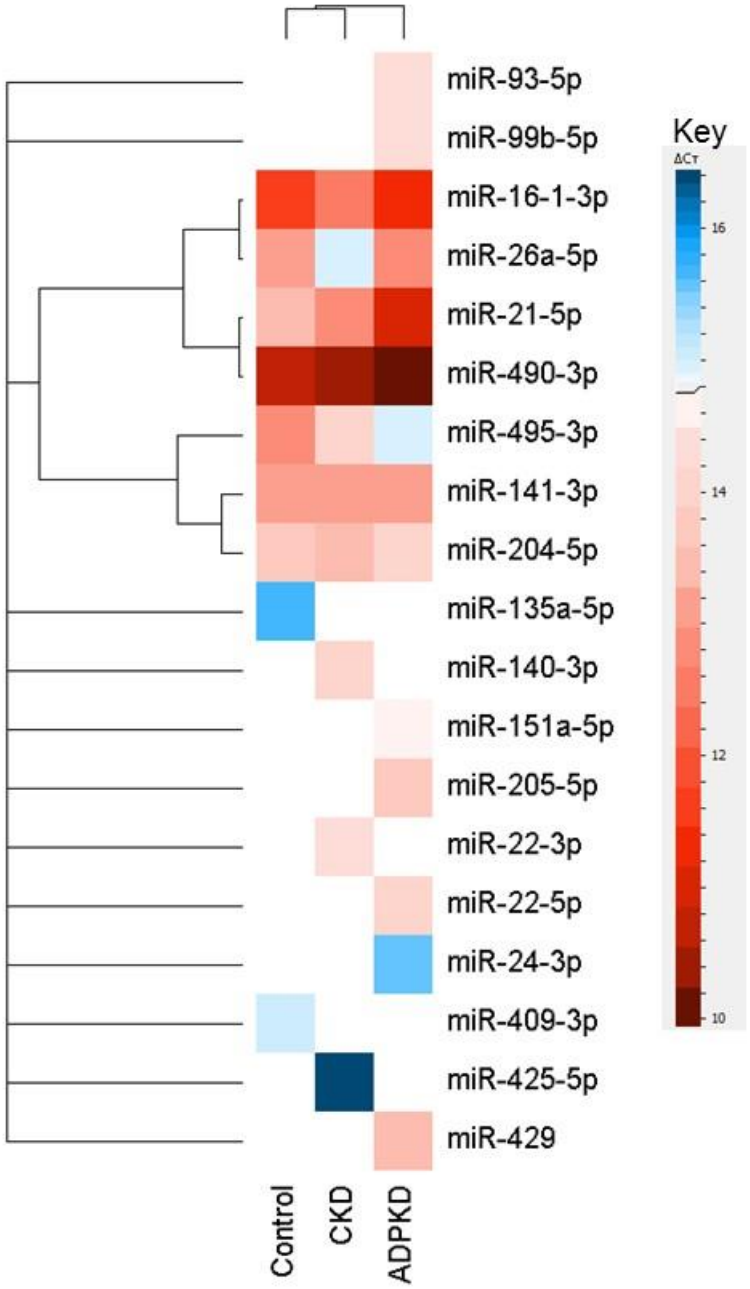

Figure 4.2. Heat map of urinary microRNAs screening using qPCR

Hierarchical clustering heat map of universally and differentially expressed miRNAs between 3 groups, healthy controls, patients with ADPKD and patients with CKD. The groups are shown on the bottom. The miRNA species are shown on the right. Each row represents a microRNA and each column represents a group. Colour key at the left illustrates the relative expression level of a microRNA (Delta CT value) across all groups; white fields indicate undetected miRNA, red indicates high expression of miRNA, and cobalt indicates relatively low expression of miRNA.

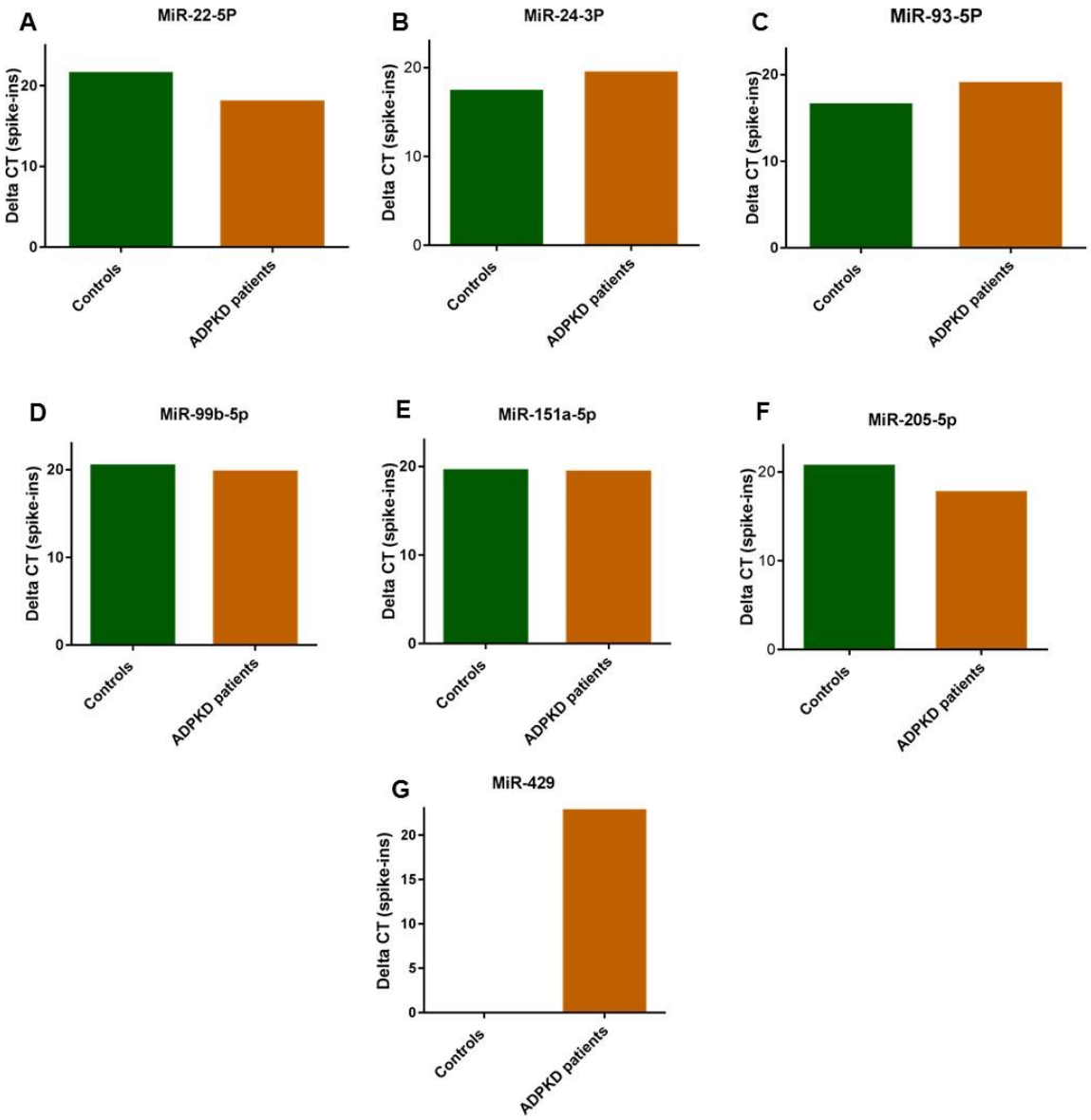

Figure 4.3. Cell free microRNAs validation

The figures show the expressions of (**A**) mir-22-5p, (**B**) mir-24-3P, (**C**) mir-93-5P, (**D**) miR-99-5P, (**E**) mir-151a-5p, (**F**) mir-205-5P and (**G**) mir-429 in urine samples from patients with ADPKD (n = 5) and healthy controls (n = 5). Delta CT values were calculated relative to spike-ins. Experiments were repeated 3 times for each primer. Mann-Whitney U test was used to analysis the data. Data was presented as mean ± SEM, and P values < 0.05 were considered statistically significant**.**

4.6 Identification of ADPKD specific exosome associated microRNA

In view of the low abundance of microRNAs in cell free urine, I next explored whether exosomes could be an alternative source of urinary microRNAs. Non-coding RNA and coding RNA have been previously reported in exosomes (Valadi, Ekstrom et al. 2007; Sato-Kuwabara, Melo et al. 2015). The use of exosome associated microRNA as a diagnostic or prognostic tool in clinical practice has shown promise based on the following factors: (1) the discovery that exosome associated microRNA are stably expressed in urine (Lv, Cao et al. 2013; Zhang, Li et al. 2015); (2) the expression of microRNAs is relatively tissue specific (Li, Yong et al. 2010); (3) exosomes are enriched with intact microRNAs compared to cell free urine or cellular microRNAs (Cheng, Sharples et al. 2014; Cheng, Sun et al. 2014; Perez-Hernandez, Forner et al. 2015); (4) exosome associated RNA represents RNA from various renal segments and tubules, whereas, RNA obtained by renal biopsy represents only the corresponding tissue at the biopsy site (Miranda, Bond et al. 2010). Differential speed centrifugation is the gold standard method for exosome isolation (Gonzales, Pisitkun et al. 2008; Fernandez-Llama, Khositseth et al. 2010; Livshits, Khomyakova et al. 2015), and produces the highest quality exosome associated RNA (Cheng, Sun et al. 2014).

4.7 Next generation sequencing (NGS)

NGS is a powerful high-throughput technique, which has the capacity to sequence millions of independent transcripts simultaneously (Shendure, Mitra et al. 2004; Lu, Tej et al. 2005; Cheng, Quek et al. 2013). NGS does not rely on any previous sequencing information, can detect low abundant transcripts, and is cost effective (Vaz, Ahmad et al. 2010). It has been used to identify microRNA expression in a number of diseases including neuroblastoma (Schulte, Marschall et al. 2010); multiple sclerosis (Bergman, James et al. 2013); cancers (Farazi, Horlings et al. 2011; Wu, Somlo et al. 2012; Chang, Kuo et al. 2015); neurodegenerative diseases (Cheng, Quek et al. 2013); and renal diseases (Mohan, Singh et al. 2016). In this study, an Illumina NGS platform was used to identify deregulated microRNAs present in urinary exosomes of a cohort of ADPKD patients as part of a discovery phase. The workflow is summarised in **Figure 4.4**.

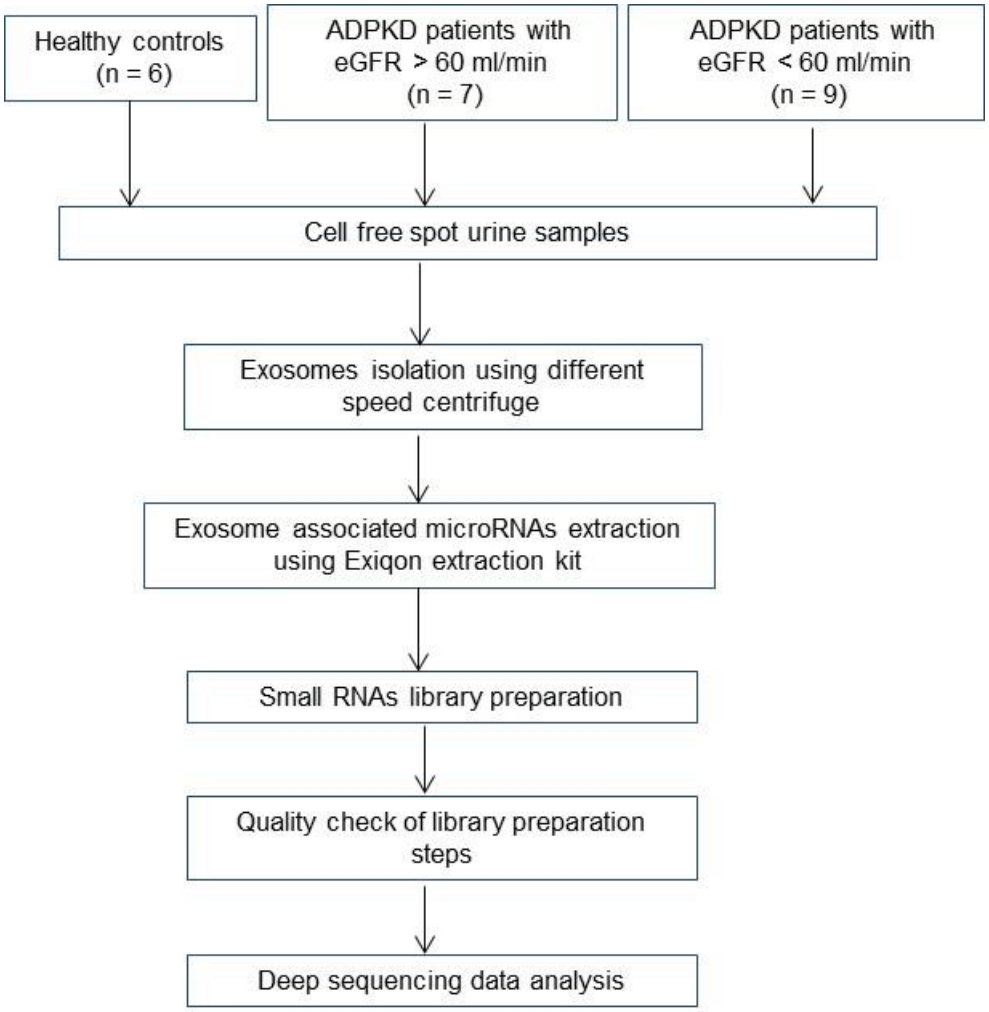

Figure 4.4. Flow chart of microRNA analysis by next generation sequencing

The flow chart of urinary exosome associated microRNA analysis by next generation sequencing in a cohort of 22 participants.

4.7.1 Baseline characteristics of the study participants

In this study, microRNA extracted from urinary exosomes from ADPKD patients with eGFR > 60 ml/min (n = 7), ADPKD patients with eGFR < 60 ml/min (n = 9) and healthy controls (n = 6) were sequenced simultaneously using an Illumina barcode technique.

The base line demographic and clinical features of the participants are displayed in **Table 4.2**. The mean ages of the study controls, ADPKD patients with eGFR > 60 ml and ADPKD patients with eGFR < 60 ml were 46.00 ± 11.64, 54.43 ± 10.98 and 53.67 ± 9.179 respectively with no significant statistically differences among the groups. There were no significant differences between the examined groups in gender, BMI, serum calcium, phosphate, cholesterol and PCR. Estimated GFR and serum uric acid were significantly higher in patients with advanced stages of ADPKD (eGFR < 60 ml/min) when compared to patients with early stages of ADPKD (eGFR > 60 ml/min) and healthy controls. In addition, there were significant differences between the 2 patient groups in mean kidney length and eGFR slopes.

Table 4.2. Clinical features of the study participants

Characteristic	Healthy controls (Mean ± SEM)	ADPKD patients with eGFR > 60 (Mean ± SEM)	ADPKD patients with eGFR < 60 (Mean ± SEM)	P value
Number	6	7	9	-
Gender (F;M)	3;3	4;3	4;5	0.881
Age (years)	46.00 ± 11.64	54.43 ± 10.98	53.67 ± 9.179	0.2987
BMI (kg/m^2)	28.22 ± 3.513	27.00 ± 2.708	25.33 ± 2.646	0.1872
eGFR (ml/min/1.73 m^2)	96.95 ± 24.55	99.71 ± 34.14	34.33 ± 17.56	< 0.0001
MKL (cm)	nm	13.28 ± 2.126	18.42 ± 2.041	0.0004
eGFR slope (ml/min/1.73 m^2)	nm	-0.3333 ± 3.470	-4.829 ± 2.105	0.0151
Calcium (mmol/l)	2.348 ± 0.04119	2.333 ± 0.05376	2.283 ± 0.07297	0.1106
Phosphate (mmol/l)	1.083 ± 0.1938	1.127 ± 0.1262	1.237 ± 0.1982	0.2437
Uric acid (µmol/l)	375.0 ± 90.40	259.0 ± 29.69	414.0 ± 133.4	0.0186
Cholesterol (mmol/l)	5.567± 0.9688	4.686 ± 0.8821	4.533 ± 0.9836	0.1259
PCR (mg/mmol)	nm	11.00 ± 1.732	24.88 ± 18.4	0.2399

The table shows the basic demographic and clinical features of the participants in the microRNAs next generation sequencing experiment. Estimated GFR was measured using CKD-EPI formula. The data was analysed using ANOVA test, a Tukey's post hoc analysis was used to find the source of insignificant. The data expressed as mean ± SEM. Abbreviations are: F; Female, M; Male, BMI; Body Mass Index, MKL; Mean Kidney Length, eGFR; estimated Glomerular Filtration Rate, PCR; Protein Creatinine Ratio, nm; not measured.

4.7.2 MicroRNAs extraction, integrity and quality check

Exosomes were isolated from total urine using a differential speed centrifugation technique and this was followed immediately by RNA extraction using Exiqon mercury's extraction columns. 50 µl of RNAs were typically obtained for downstream analysis. The purified microRNA was concentrated and input was normalised to the starting volume of urine.

After a small library preparation, it is essential to include only the nucleotides corresponding to microRNAs and eliminate all longer nucleotides that corresponding to mRNA or rRNA (Motameny, Wolters et al. 2010). To do that, the RNA samples were run on an agarose gel and bands corresponding to the known size of microRNAs were cut and included for downstream analysis. **Figure 4.5** shows the purification of microRNAs from library pool-1 obtained from pooling 12 samples. Library pool-2 which was corresponding to pooling of 10 samples was processed in the same way.

The library was then analysed on an Agilent Bioanalyzer to calculate library concentration. The final concentration of the library is important before a cluster generation, because a low concentration results in a few clusters and subsequently low sequencing reads. On the other hand, a high library concentration can result in a dense cluster which leads to complicated data analysis (Corney 2013). Therefore, before proceeding to a cluster generation, library validation was conducted to ensure accurate quantification of the library. Every peak on the Bioanalyzer created a cluster and the final morality was the result of summation of all peaks on the electropherogram (**Figure 4.6**).

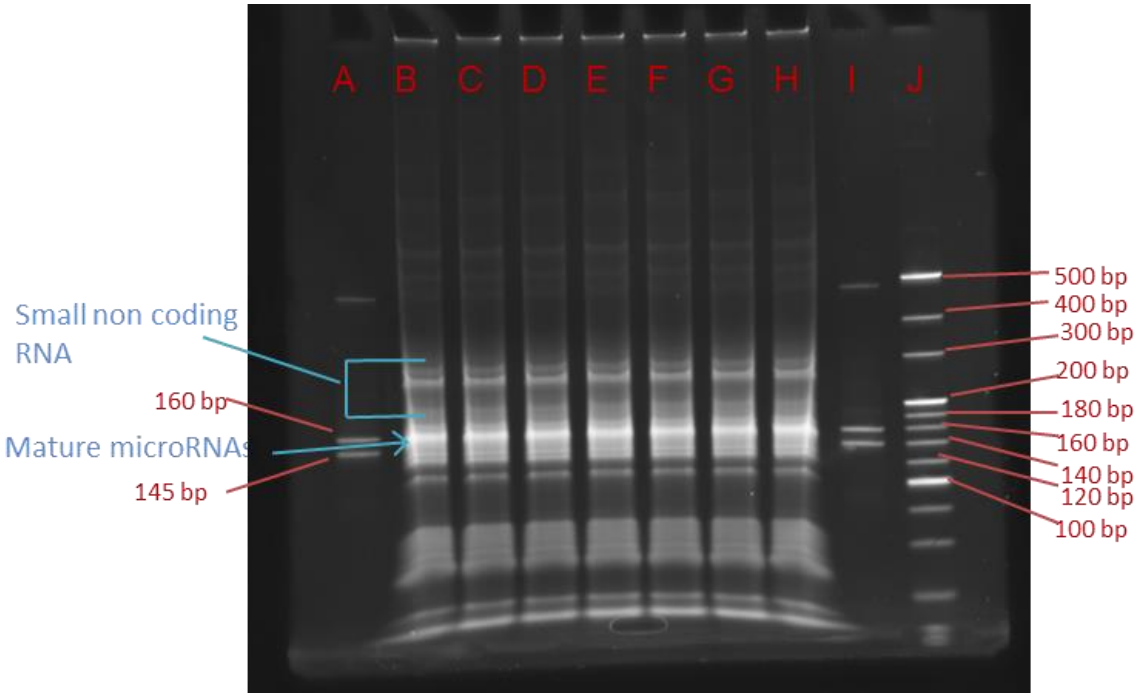

Figure 4.5. Gel analysis of a small RNA library of urine exosomes

Lane (**A**) custom RNA ladder, (**B** to **H**) a purification of small library pooling-1 (consisting of 12 samples), (**I**) custom RNA ladder (**J**) high resolution ladder were run on a 6% Novex gel, followed by visualization on a dark UV trasilluminator. Custom RNA ladder involves of 145 bp, 160 bp and 160 bp dsDNA. The 147 bp band comprises mature microRNAs. Sequencing was conducted on the pooled bands.

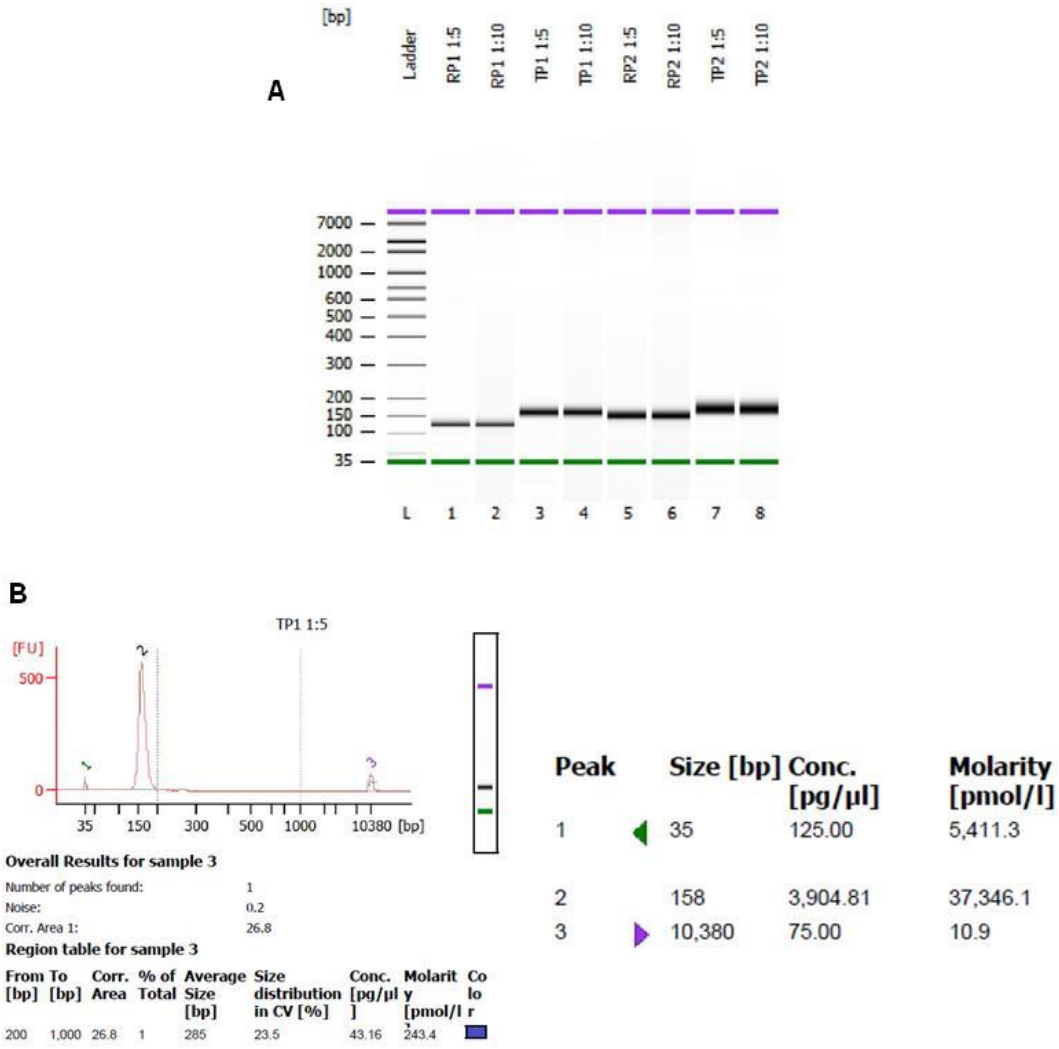

Figure 4.6. Agilent Bioanalyzer of the final small libraries

Before proceed to cluster generation, library validation was conducted to accurate quantification of the library using Agilent Bioanalyzer. (**A**) The figure represents the following: lane L; the ladder, lane 1 and 2; RNA library belonged to the neuroscience department, lane 3 and 4; library pool-1 with 2 different concentrations of 1;5 and 1;10, lane 5 and 6; RNA library belonged to the neuroscience department, lane 7 and 8; library pool-2 with 2 different concentrations of 1;5 and 1;10. 1;5 concentration provided a better result and therefore, the libraries were diluted 1;5. (**B**) The figure represents Bioanlyzer electropherograms for the library pool-1 and every peak on the electropherogram creates cluster. The final molarity is the result of summation of all peaks on the graph.

4.7.3 NGS quality control analysis

After completion of the sequencing runs, the raw data was de-multiplexed according to their corresponding specific barcode into separate samples using bcl2fastq conversion software (version1.8.4). Before proceeding to identify differentially expressing microRNAs, an overview of the data quality was performed. The quality of generated data was checked using the default settings of Strand NGS (**Figure 4.7**). The quality check of FASTQ data is based on a PHRED quality score (Ewing and Green 1998; Ewing, Hillier et al. 1998; Cock, Fields et al. 2010). Phred equation converts the quality score into a probability of error value; Q score $= -10 \log_{10} p$ (where p = estimated probability of base call) so a Q score of 10 represents 90% probability of errors (Brockman, Alvarez et al. 2008; Gabaldón and Alioto 2016). According to Phred's equation, all the sequencing reads from my study had a quality score > 30, and that denoted that the base call accuracy of overall reads is 99.9% (**Figure 4.7A**). The size distributions of sequencing reads were evaluated for all samples. The length distribution of microRNAs is around 19-24 nt (Li, Yong et al. 2010; Wongwarangkana, Fujimori et al. 2015). **Figure 4.7B** shows that our read size distribution was mainly around this range.

A total of 74.22% of the mean sequencing reads were mapped to the human genome (hg39), the percentage of alignment for each sample and the number of ignored reads are shown in **Table 4.3**. The unaligned reads were discarded from downstream analysis. Unaligned reads are thought to be mainly due to either internal modification or variation (adenosine deaminase acting on RNA editing or single nucleotide polymorphisms), base-calling errors, post transcription modification or contamination (Vitsios and Enright 2015). Ignoring of sequence reads are due to their small size or absence of the adapter.

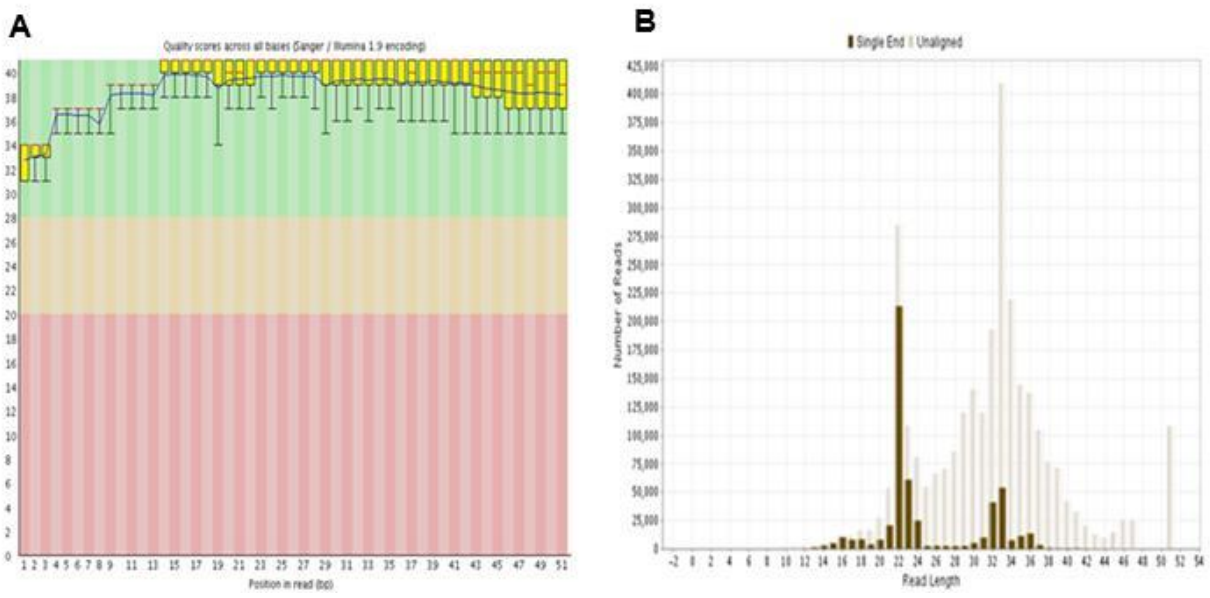

Figure 4.7. Quality control of sequenced reads

(**A**) The figure shows Q score for a represented sample generated by Strand NGS algorithm. The elements of the figure are as following; X axis represents the base position in sequenced read, y axis represents the quality score, the yellow boxes and whiskers denote 25-75% interquartile range and the 10 and 90% points respectively, the red and blue lines represent the median and the mean of the read bases position respectively. Higher quality score has better base call. The representing plot shows that all mean per base quality score are > 32.5 and the medians are around 39. (**B**) The graph shows a representative of nucleotides distribution of exosomes associated small RNA in a spot urine sample. The length distribution of sequenced read was concentrated mainly around 22 nt.

Table 4.3. Summary of NGS reads number and alignment

Samples	Total number of reads	Aligned reads	Unaligned reads	Ignored reads
HV-117	2,844,987 (100%)	2,319,351 (81.50%)	519,907 (18.30%)	5729 (0.2%)
HV-118	3,041,444 (100%)	2,400,023 (78.90%)	360,937 (11.90%)	280484 (9.2%)
HV-122	867,712 (100%)	740,828 (85.40%)	120,136 (13.80%)	6748 (0.8%)
HV-124	11,240,245 (100%)	8,593,390 (76.50%)	2,642,323 (23.50%)	0%
HV-125	4,399,811 (100%)	3,535,367 (80.40%)	855,480 (19.40%)	8964 (0.2%)
HV-129	8,846,375 (100%)	6,890,841 (77.90%)	1,951,444 (22.10%)	0%
ADPKD-13	714,710 (100%)	639,678 (89.50%)	71,207 (10%)	3825 (0.5%)
ADPKD-26	326,238 (100%)	295,123 (90.50%)	16,049 (4.90%)	15060 (4.6%)
ADPKD-28	1,390,916(100%)	1,224,563 (88%)	161,866 (11.60%)	4487 (0.4%)
ADPKD-30	3,124,067(100%)	2,426,669 (77.70%)	671,089 (21.50%)	262313 (0.8%)
ADPKD-35	5,716,313(100%)	4,619,778 (80.80%)	1,088,547 (19%)	7988 (0.2%)
ADPKD-71	10,098,773 (100 %)	7,800,449 (77.20%)	2,293,850 (22.70%)	4474 (0.1%)
ADPKD-16	3,202,931(100%)	1,699,184 (53.10%)	1,492,710 (46.60%)	11037 (0.3%)
ADPKD-34	8,546,602(100%)	5,847,857 (68.40%)	2,653,050 (31%)	45695 (0.6%)
ADPKD-60	714,535 (100%)	514,018 (71.90%)	191,046 (26.70%)	9471 (1.4%)
ADPKD-76	515,138(100%)	352,034 (68.30%)	147,870 (28.70%)	15234 (3%)
ADPKD-69	1,434,519 (100%)	1,037,329 (72.30%)	386,167 (26.90%)	11023 (0.8%)
ADPKD-47	3,184,642 (100%)	2,234,486 (70.20%)	910,131 (28.60%)	40025 (1.2%)
ADPKD-59	8,980,670 (100%)	5,562,089 (61.90%)	3,383,031 (37.70%)	35550 (0.4%)
ADPKD-99	340,938 (100%)	222,672 (65.30%)	66,564 (19.50%)	51702 (15.2%)
ADPKD-43	124,176 (100%)	66,542 (53.60%)	37,799 (30.40%)	86310.45 (16%)
ADPKD-96	1,499,333 (100%)	952,570 (63.50%)	489,576 (32.70%)	57187 (3.8%)

4.7.4 Differential expression microRNAs

Differential expression analysis was used to determine the relative expression levels of mapped mature microRNAs. Firstly the raw counts were normalised using DESeq (Anders and Huber 2010). This normalisation approach relies on a calculation of a geometric mean across all samples, and for each sample, the expression of a particular transcript is divided by the geometric mean (Anders and Huber 2010; Dillies, Rau et al. 2013; Gunawan, Sahadevan et al. 2013). DESeq normalization is widelly used in next generation sequencing data (Chang, Thomas et al. 2013; Dillies, Rau et al. 2013; Barsanti, Trivella et al. 2015; Hutchins, Eckalbar et al. 2016).

Three separate microRNA sequencing packages were used to analyse the next generation sequencing data including Strand NGS, Illumina's BaseSpace and Chimira (section 1.11.10). The 3 packages aligned the next generation sequencing data to a same reference (hg39) and also normalize the data using DESeq. Strand NGS is an integrated pipeline provides a comprehensive analysis of microRNAs sequenced data and it has been applied widely in the literature (Dasgupta, Xu et al. 2015; Farr, Januszewski et al. 2015; Ignacio, Hicks et al. 2015; Chen, Huang et al. 2016). Illumina's BaseSpace and Chimira are online pipelines offer analysis of next generation sequencing RNA data including microRNAs (Krampis and Wultsch 2015; Van Neste, Gansemans et al. 2015; Vitsios and Enright 2015).

Each program identified a number of different microRNAs. Strand NGS identified 22 microRNAs; Illumina identified 28 microRNAs; Chimira identified 14 microRNAs. 6 microRNAs were commonly identified by Illumina and Chimira programs. 7 common differentially expressed microRNAs identified by all 3 packages are shown in **Figure 4.8** and **Table 4.4**.

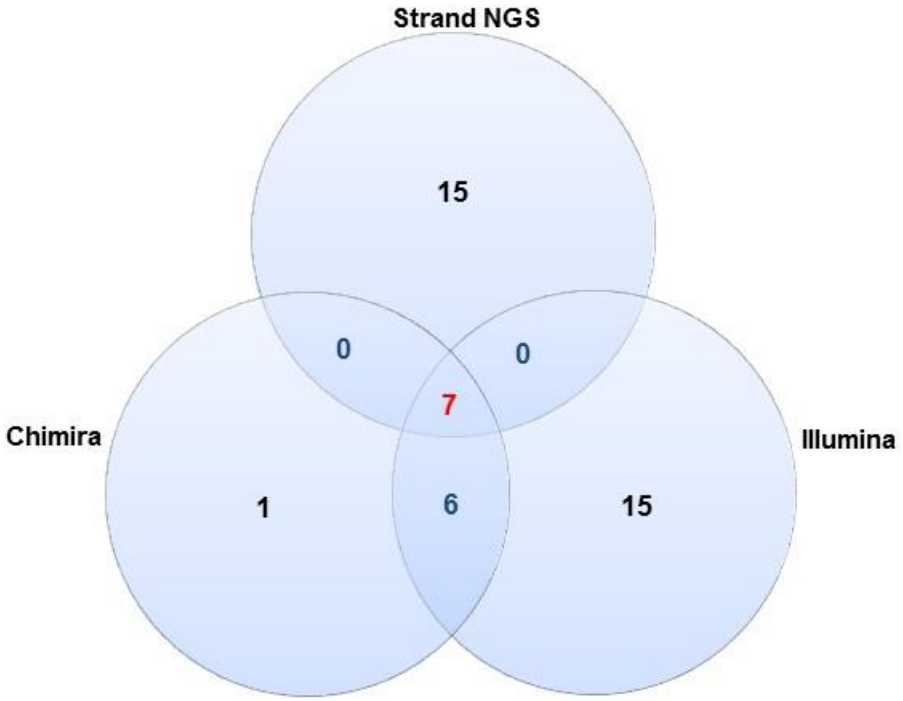

Figure 4.8. Exosomal microRNAs yielded by the 3 algorithm

Venn diagram shows common and unique urinary exosomal associated microRNAs identified by the 3 different algorithms. Red line shows microRNAs identified by the 3 algorithms. Blue lines indicate microRNAs identified by two algorithms. black lines show microRNAs identified by only one algorithm.

Table 4.4. Significantly altered microRNAs by NGS

Strand NGS		Illumina		Chimira	
MiRNA	Fold changes	MiRNA	Log Fold changes	MiRNA	Fold changes
MiR-25	-1.175	MiR-16-5p	-1.262	MiR-27b-3p	/
MiR-26b	1.313	MiR-21-5p	-1.07	MiR-30a-5p	/
MiR-27b	-2.5	MiR-22-3p	-0.9122	MiR-30d-5p	/
MiR-30a	-1.956	MiR-27a-3p	-1.636	MiR-30e-5p	/
MiR-30b	-1.476	MiR-27b-3p	-1.355	MiR-148a-3p	/
MiR-30d	-1.582	MiR-30a-5p	-0.865	MiR-181a-5p	/
MiR-30e	-2.08	MiR-30d-5p	-0.53	MiR-192-5p	/
MiR-93	1.456	MiR-30e-5p	-1.2	MiR-194-5p	/
MiR-99a	-1.371	MiR-101-3p	-0.895	MiR-205-5p	/
MiR-130a	-1.8642	MiR-148a-3p	-1.211	MiR-210-3p	/
MiR-140	-2	MiR-181a-5p	-0.89	MiR-22-3p	/
MiR-148a	-1.691	MiR-184	-2.37	MiR-378a-3p	/
MiR-192	-2	MiR-186-5p	-0.757	MiR-378d	/
MiR-194-1	-4	MiR-192-5p	-0.963	MiR-4532	/
MiR-194-2	-4	MiR-193b-3p	-1.758		
MiR-197	3.369	MiR-194-5p	-0.964		
MiR-424	-1.56	MiR-203a-3p	-2.316		
MiR-454	1.355	MiR-205-5p	-3.163		
MiR-577	-1.81	MiR-210-3p	-1.67		
MiR-589	1.361	MiR-221-5p	-1.72		
MiR-891a	-2.678	MiR-378a-3p	-1.387		
MiR-1307	-2.49	MiR-769-5p	-2.1		
		MiR-891a-5p	-2		
		MiR-1273f	-1.5		

		MiR-1273h-5p	-1.8		
		MiR-3665	-1.84		
		MiR-4532	-2.73		
		MiR-6884-3p	-3		

The table displays the commonly identified differentially expressed microRNAs by the 3 algorithms with P values < 0.05. Red represent commonly identified microRNAs, while, green represent microRNAs identified by 2 algorithms. Of notes, Chimira does not provide fold changes.

4.8 Validation of deregulated microRNAs

Out of differentially expressed microRNAs commonly identified by the 3 algorithms, I focused on microRNAs from a same family or located on a same cluster to be further validated in a cohort study of 60 participants using TaqMan qPCR assays. I selected 3 members of miR-30 family (including miR-30a-5p, miR-30d-5p and miR-30e-5p); given that these members have similar 5' terminal seed sequences and are located in pairs on 3 different chromosomes (Jiang, Qiu et al. 2013). The miR-30 family is highly expressed in renal tissue and is required for pronephric development (Agrawal, Tran et al. 2009; Hand, Master et al. 2009). Of interest, a set of microRNAs including a member of miR-30 was reported to be involved in transcriptional reprogramming at the time of cystogenesis in a *PKD1*$^{-/-}$ mouse model (Pandey, Qin et al. 2011). MiR-192-5p and miR-194-5p were also selected for validation as both are highly expressed in the kidney; they are also located on same cluster of chromosomes 11 with 109-bp distance between them suggesting a common regulation as a transcription unit (Sun, Koo et al. 2004; Pichiorri, Suh et al. 2010; Park, Woo et al. 2011). Finally, miR-193b-3p was studied as it was identified independently from a parallel microarray study in our laboratory to be downregulated in a panel of human ADPKD cystic cell lines (Streets, Magayr et al. 2017). However, significant dysregulation of miR-193b-3p in urine exosomes was only identified by Strand NGS software. **Figure 4.9** shows the workflow of the validation experiments.

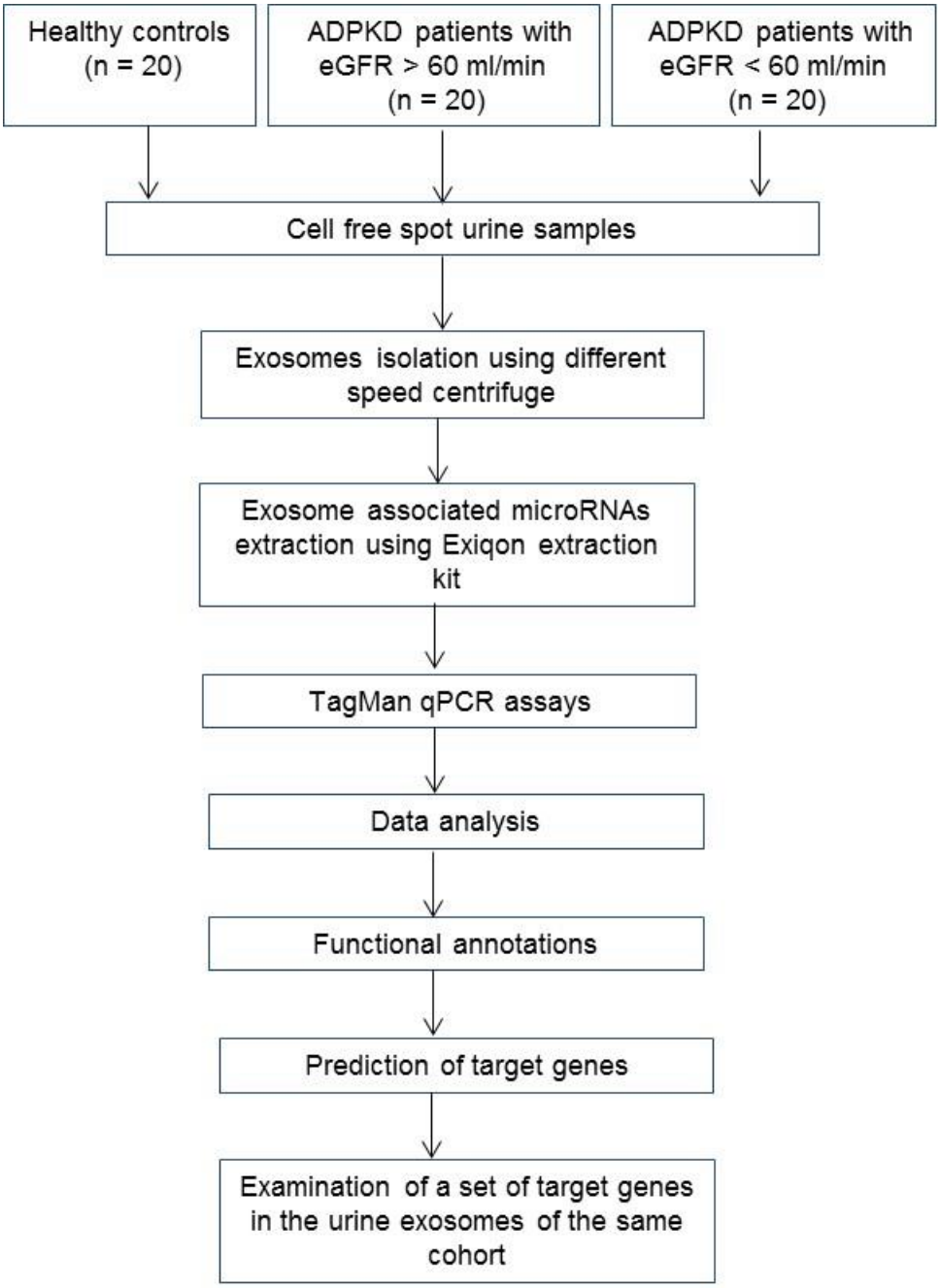

Figure 4.9. Flow chart for validation step of the results of microRNAs screening phase

The graph shows the flow chart of urine exosomes associated microRNAs validation phase in a cohort of 60 participants.

4.8.1 Baseline characteristics of the study participants

A total of 60 participants were selected from our total cohort based on age and gender matching, eGFR measurement at the time of recruitment and the availability of sufficient urine samples. The differentially expressed microRNAs were examined in ADPKD patients with eGFR > 60 ml/min (n = 20), ADPKD patients with eGFR < 60 ml/min (n = 20) and healthy controls (n = 20). The sample size of 40 patients was calculated based on an eGFR slope > 3 ml/min over 5 years, and it provided 80% power to detect an odds ratio of 2.5 and type 1 error (alpha level) of 0.05 using z test. The base line clinical characteristics of this cohort are depicted in **Table 4.5**. Samples used in the next generation sequencing experiment were also included in these experiments.

There were no significant differences in the mean age, gender, serum calcium, phosphate, cholesterol and uric acid between the groups. However, there was a significant difference in the eGFR at the time of recruitment as well as BMI and PCR between the 3 groups. In addition, there were significant differences between the 2 patient groups in mean kidney length and the eGFR slope.

Table 4.5. Clinical features of the study participants

Characteristic	Healthy controls (Mean ± SEM)	ADPKD patients with eGFR > 60 (Mean ± SEM)	ADPKD patients with eGFR < 60 (Mean ± SEM)	P value
Number	20	20	20	-
Gender (F;M)	10;10	10;10	8;12	0.765
Age (years)	46.15 ± 12.62	47.80 ± 11.79	54.00 ± 9.830	0.0829
BMI (kg/m^2)	25.94 ± 3.475	30.12 ± 4.554	26.56 ± 3.974	0.0064
eGFR (ml/min per 1.73 m^2)	92.16 ± 23.25	95.45 ± 24.47	34.80 ± 12.64	< 0.0001
MKL (cm)	nm	13.82 ± 2.243	18.61 ± 3.440	< 0.0001
eGFR slope (ml/min per 1.73 m^2)	nm	-0.2429 ± 3.283	-3.688 ± 2.085	0.0017
Calcium (mmol/l)	2.320 ± 0.07152	2.333 ± 0.06789	2.291 ± 0.08811	0.2232
Phosphate (mmol/l)	1.097 ± 0.1547	1.097 ± 0.1547	1.157 ± 0.1945	0.4468
Uric acid (µmol/l)	311.0 ± 74.15	327.3 ± 90.32	390.5 ± 136.5	0.0559
Cholesterol (mmol/l)	5.330 ± 0.9836	5.211 ± 1.149	4.675 ± 0.8735	0.1001
PCR (mg/mmol)	nm	11.08 ± 6.487	25.47 ± 21.82	0.0361

The table shows the basic demographic and clinical features of the participants in the validation experiments. Estimated GFR was measured using CKD-EPI formula. Data was analysed using one-way ANOVA test followed by a Tukey's post hoc analysis. Data expressed as mean ± SEM. Red lines highlight significant P value (< 0.05). Abbreviations are: F; Female, M; Male, BMI; Body Mass Index, MKL; Mean Kidney Length, eGFR; estimated Glomerular Filtration Rate, PCR; Protein Creatinine Ratio, nm; not measured.

4.8.2 Identification of suitable housekeeping genes

Identification of a stable expressed endogenous microRNA is a key strategy for successful qPCR validation. NormFinder was used to identify suitable universally expressed microRNAs in the sequenced samples (Andersen, Jensen et al. 2004). NormFinder is used to assess the stability of differentially expressed microRNA based on their expression variability and stability values, and it is available at http://moma.dk/normfinder-software. The result of NormFinder analysis is shown in **Table 4.6**.

MiR-191-5p was the most universally expressed microRNAs among the samples with a stability value of 94.714. Interestingly, ThermoFisher scientific suggests this microRNA as one of their microRNAs expression controls. In addition, miR191-5p has been shown to be the most stably expressed microRNA in 13 normal tissue and 5 cancer tissues using GeNorm and NormFinder (Peltier and Latham 2008). In addition, it has been used as an endogenous control in a urine microRNAs study (Egidi, Cochetti et al. 2015). In our project, miR-191-5p was universally expressed with no significant difference between urine exosome samples from ADPKD patients with eGFR > 60 ml/min, ADPKD patients with eGFR < 60 ml/min and healthy controls (**Figure 4.10 A**).

Use of multiple microRNAs as reference genes for normalization of qPCR data is an acceptable approach in the literature (Mestdagh, Van Vlierberghe et al. 2009). A cel-miR-39-3p spike-ins has been also used in my study as normalization for technical variation in combination with an endogenous control. The spike–ins normalisation strategy has been used by a number of previous studies (Perez-Hernandez, Forner et al. 2015; Sohn, Kim et al. 2015; Li, Ma et al. 2016). In our project, the recovery of cel-miR-39-3p was not significantly different between urine samples from healthy controls, ADPKD patients with eGFR > 60 ml/min and ADPKD patients with eGFR < 60 ml/min (**Figure 4.10 B**). Therefore, to validate the expression of microRNAs in urinary exosomes, delta Ct values were calculated relative to urinary expression of miR-191-5p (an endogenous control) and cel-miR-39-3p (an exogenous control).

Table 4.6. NormFinder result

Gene name	Stability value
miR-191-5p	94.719
miR-204-5p	107.050
miR-99b-5p	112.576
miR-3960	119.121
let-7a-5p	122.965
miR-30a-5p	123.898
miR-30a-3p	133.920
miR-92a-3p	135.363
miR-146b-5p	138.338
miR-143-3p	140.055
miR-30d-5p	140.170
miR-26b-5p	140.338
miR-103a-3p	140.894
miR-30c-5p	140.978
miR-151a-3p	140.981
miR-99a-5p	141.347
miR-4532	141.353
miR-25-3p	141.399
let-7i-5p	141.627
miR-19b-3p	141.753

The table shows the top 20 microRNAs ranked according to their stability values as calculated by NormFinder. A lower stability value denotes a more stable microRNA. MiR-191-5p showed the best stability among the examined microRNAs.

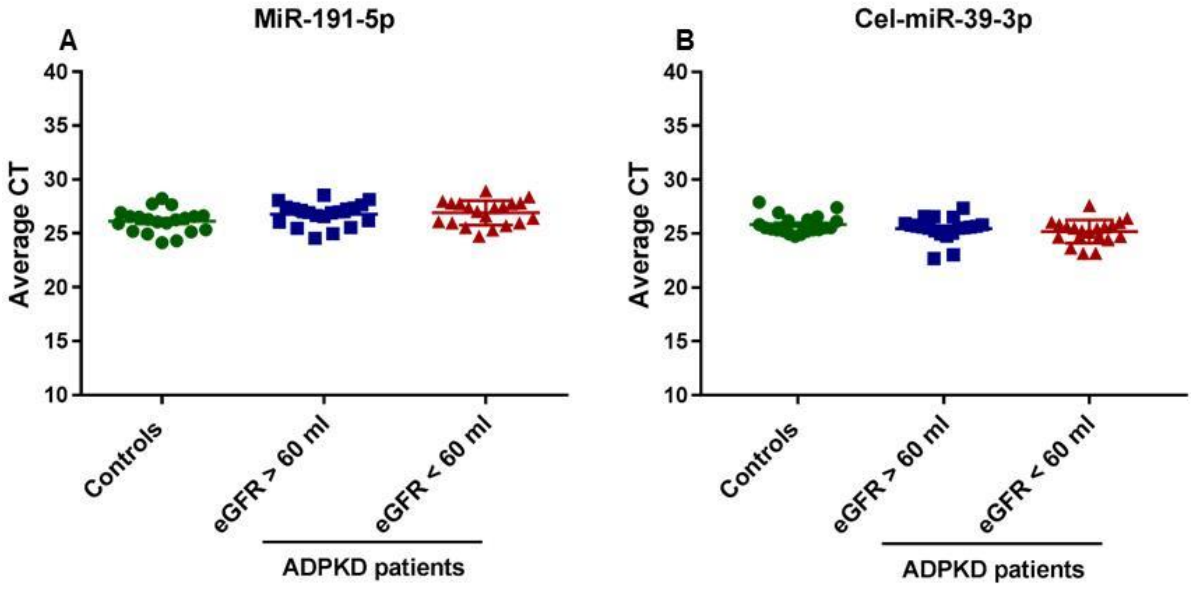

Figure 4.10. Stability of the endogenous and exogenous microRNAs expression controls in urinary exosomes

The figure shows the expressions of (**A**) the endogenous control (miR-191-5p) and (**B**) the exogenous control (cel-miR-39-3P) as determined by qPCR in healthy controls (n = 20), patients with eGFR > 60 ml/min (n = 20) and patients with eGFR < 60 ml/min (n = 20). One-way ANOVA revealed no statistically difference in urinary expressions of miR-191-5p or cel-miR-39-3P between the examined groups.

4.8.3 Analysis of differentially expressed urinary microRNAs in patients with ADPKD and healthy controls

Firstly, the expression levels of differentially expressed microRNAs were examined in urine exosomes from patients with ADPKD (n = 40) and matched healthy controls (n = 20) using TaqMan qPCR assays. I found that, in agreement with the differential expression analysis of the next generation sequencing results, differentially expressed microRNAs were significantly decreased (except miR-30a-5p) in urine exosomes from patients with ADPKD compared to healthy controls (**Figure 4.11A** to **F**). Fold change expression differences of miR-30a, miR-30d, miR-30e, miR-192-5p, miR-193b-3p and miR-194-5p in urine samples from the patients group compared to the controls group were 0.74, 0.71, 0.59, 0.39, 0.8 and 0.46, respectively.

Secondly, to determine whether there was a difference in microRNA expression in patients with early stages ADPKD (eGFR > 60 ml/min) (n = 20) compared to late stage disease (eGFR < 60 ml/min) (n = 20) and healthy controls (n = 20). One-way ANOVA followed by a Tukey's post hoc analysis was used to analyse the qPCR data. Since one-way ANOVA test showed significant statistical results between the groups, a Tukey's post hoc analysis was conducted to find the source of significance (**Figure 4.12A** to **F**). Among the differentially expressed microRNAs studied, only mir-192-5p showed a significant decrease in ADPKD patients with eGFR > 60 ml/min when compared to healthy controls (fold change 0.54) (**Figure 4.12D**). However, all differentially expressed microRNAs showed significant reductions in ADPKD patients with eGFR < 60 ml/min when compared to healthy controls or to ADPKD patients with eGFR > 60 ml/min (except miR-193b-3p) (**Figure 4.12A** to **F**). Fold change expression differences of miR-30a, miR-30d, miR-30e, miR-192-5p, miR-193b-3p and miR-194-5p in urine samples from the patients with eGFR < 60 ml/min compared to healthy controls were 0.45, o.41, 0.36, 0.25, 0.64 and 0.31, respectively.

Thirdly, I compared the expression of the differentially expressed microRNAs in patients with a rapidly progressive disease (eGFR slope > 3 ml/min over 5 years) (n = 22), patients with a slowly progressive disease (eGFR slope < 3 ml/min over 5 years) (n = 17) and healthy controls (n = 20) using one-way ANOVA followed by a Tukey's post hoc analysis. Among the differentially expressed microRNAs, only mir-192-5p showed a significant decrease in urine samples from ADPKD patients with eGFR slope < 3 ml/min over 5 years as compared to healthy controls (**Figure 4.13D**). All differentially expressed microRNAs showed significant reductions in urine samples from ADPKD patients with eGFR slope > 3 ml/min over 5 years when compared to healthy controls

or to the patients with eGFR slope < 3 ml/min (except miR-194-5p) (**Figure 4.13 A** to **F**).

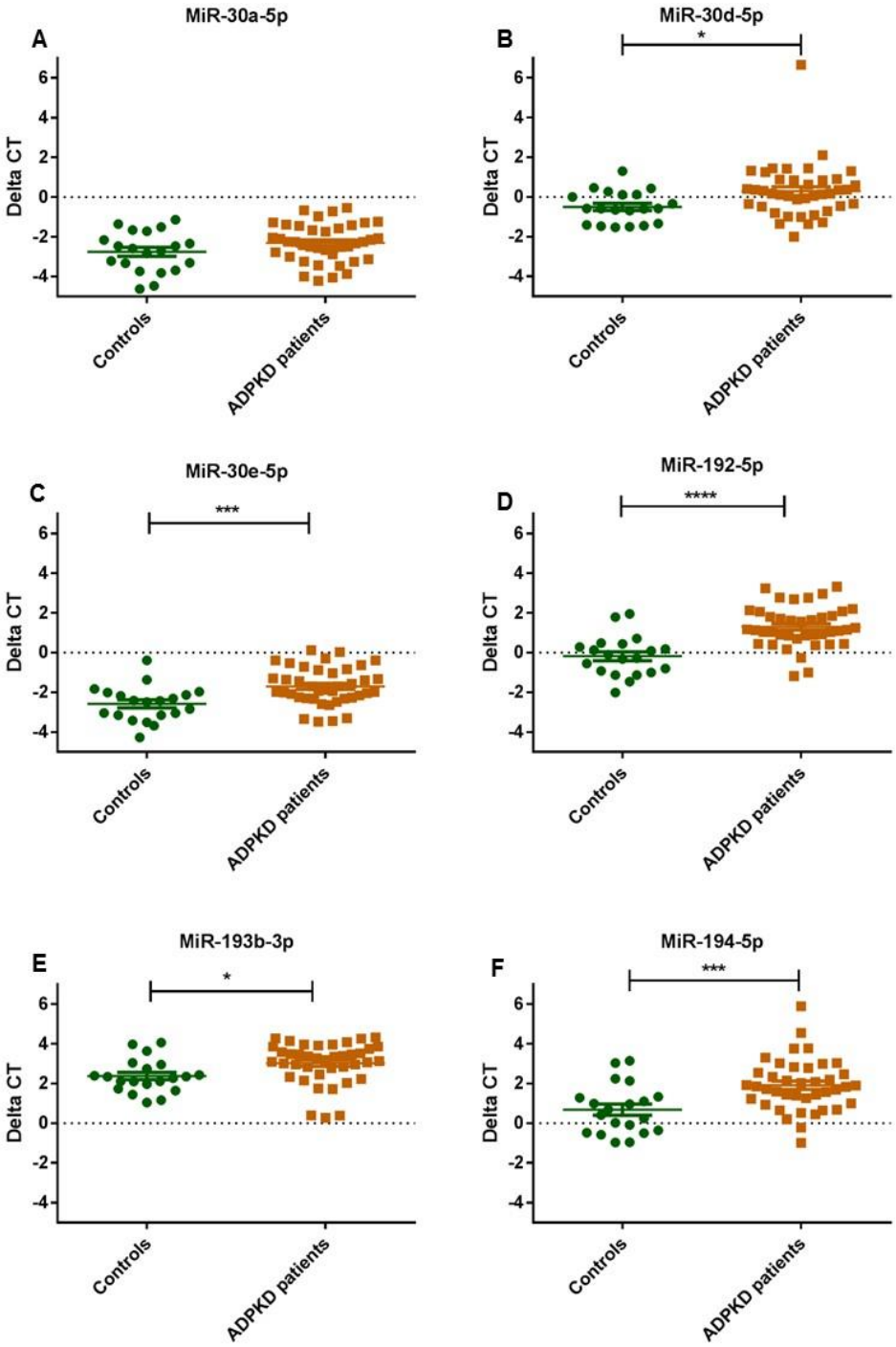

Figure 4.11. Comparison of urinary microRNA expressions in ADPKD patients and healthy controls

The figure shows the expressions of urine exosomes associated (**A**) miR-30a-5p, (**B**) miR-30d-5p, (**C**) miR-30e-5p, (**D**) miR-192-5p, (**E**) miR-193b-3p and (**F**) miR-194-5p as determined by qPCR in healthy controls (n = 20) and patients with ADPKD (n = 40). Corrected results, to endogenous (miR-191-5p) and exogenous controls (cel-miR-39-3p), are display in the graph. P value > 0.05 was considered non-significant, * denotes P value < 0.05; ** denotes P value < 0.01; *** denotes P value < 0.001 and **** denotes P value < 0.0001. Data is displayed as mean ± SEM.

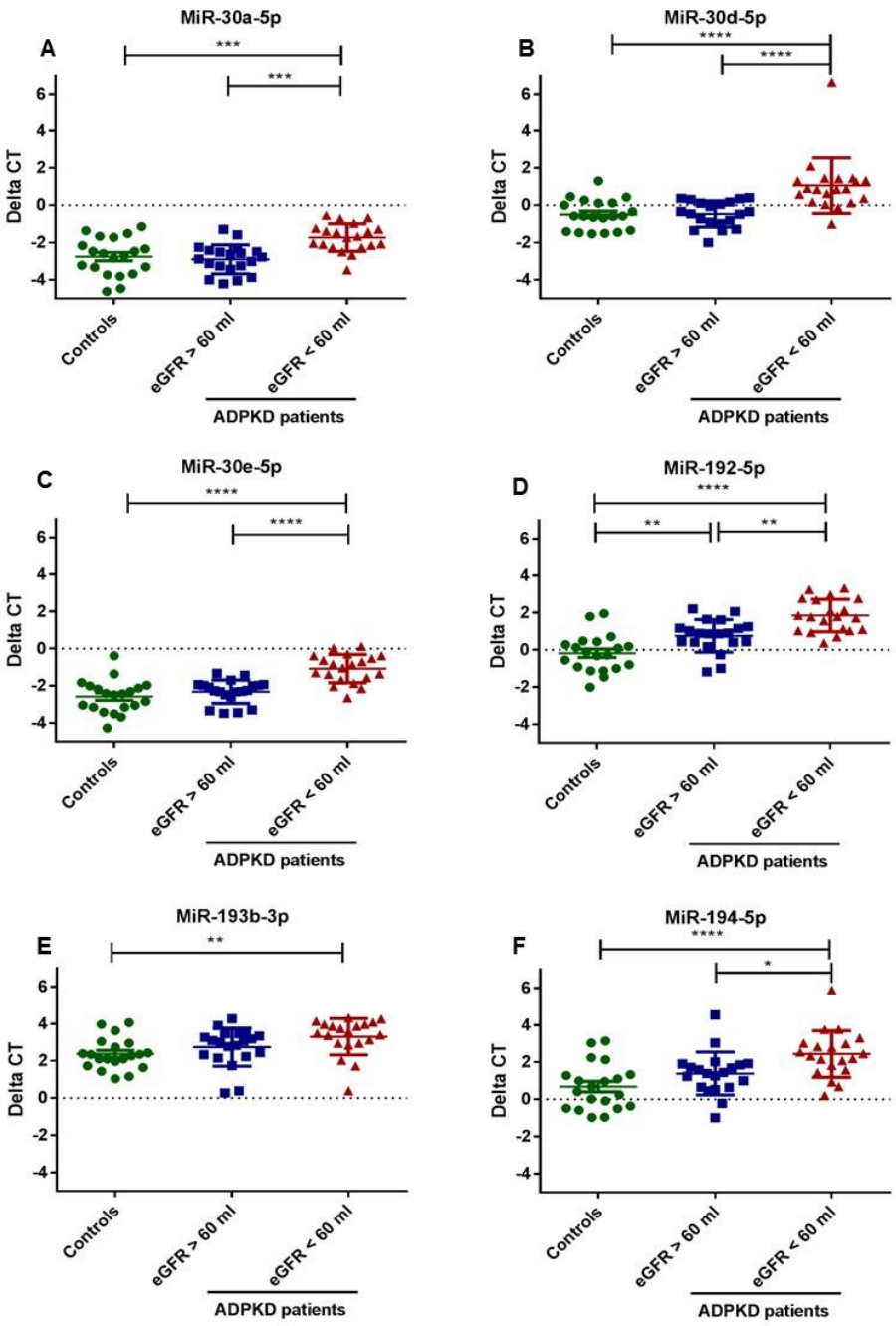

Figure 4.12. Comparison of differentially expressed microRNAs in urine of ADPKD patients with eGFR > 60 ml/min, ADPKD patients with eGFR < 60 ml/min and healthy controls

The figure shows the expressions of urine exosomes associated (**A**) miR-30a-5p, (**B**) miR-30d-5p, (**C**) miR-30e-5p, (**D**) miR-192-5p, (**E**) miR-193b-3p and (**F**) miR-194-5p as determined by qPCR in healthy controls (n = 20), patients with eGFR > 60 ml/min (n = 20) and patients with eGFR < 60 ml/min (n = 20). Corrected results, to endogenous (miR-191-5p) and exogenous controls (cel-miR-39-3p), are display in the graph. P value > 0.05 was considered non-significant, * denotes P value < 0.05; ** denotes P value < 0.01; *** denotes P value < 0.001 and **** denotes P value < 0.0001. Data is displayed as mean ± SEM.

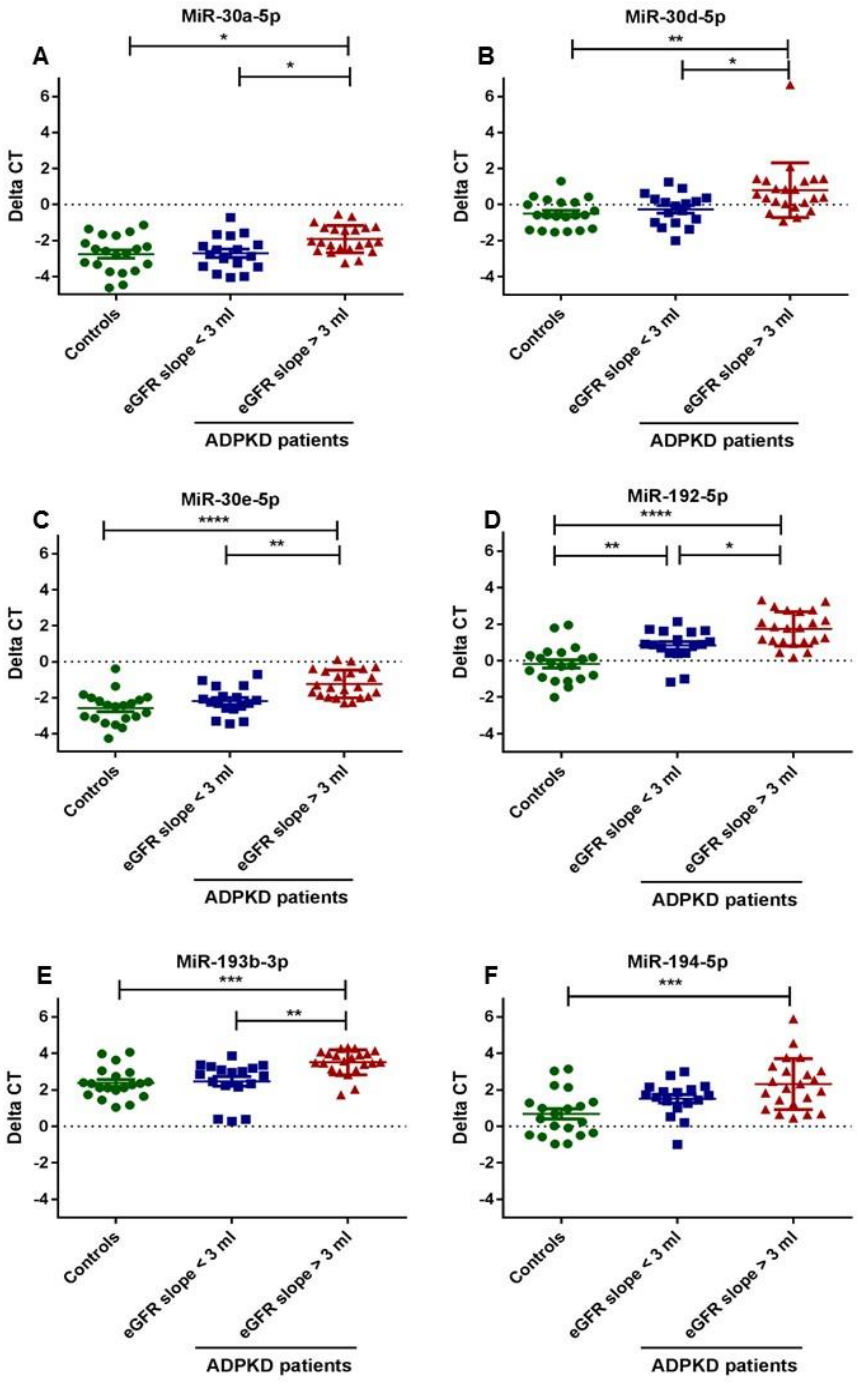

Figure 4.13. Comparison of differentially expressed microRNAs in urine of ADPKD patients with eGFR slope < 3 ml/min, ADPKD patients with eGFR slope > 3 ml/min and healthy controls

The figure shows the expressions of urine exosomes associated (**A**) miR-30a-5p, (**B**) miR-30d-5p, (**C**) miR-30e-5p, (**D**) miR-192-5p, (**E**) miR-193b-3p and (**F**) miR-194-5p as determined by qPCR in healthy controls (n = 20), patients with eGFR slope < 3 ml/min (n = 17) and patients with eGFR slope > 3 ml/min (n = 22). Corrected results, to endogenous (miR-191-5p) and exogenous controls (cel-miR-39-3p), are display in the graph. P value > 0.05 was considered non-significant, * denotes P value < 0.05; ** denotes P value < 0.01; *** denotes P value < 0.001 and **** denotes P value < 0.0001. Data is displayed as mean ± SEM.

4.8.4 Association of differentially expressed urinary microRNAs with conventional measures of ADPKD

Correlation of the microRNA expression with conventional renal diagnostic tests including eGFR and MKL was evaluated using a Spearman-correlation test. Associations of differentially expressed microRNAs with eGFR and MKL are depicted in **Figure 4.14** and **Figure 4.15**.

All the microRNAs studied showed negative correlations with eGFR and positive correlations with MKL indicating that microRNA expression decreased with disease progression.

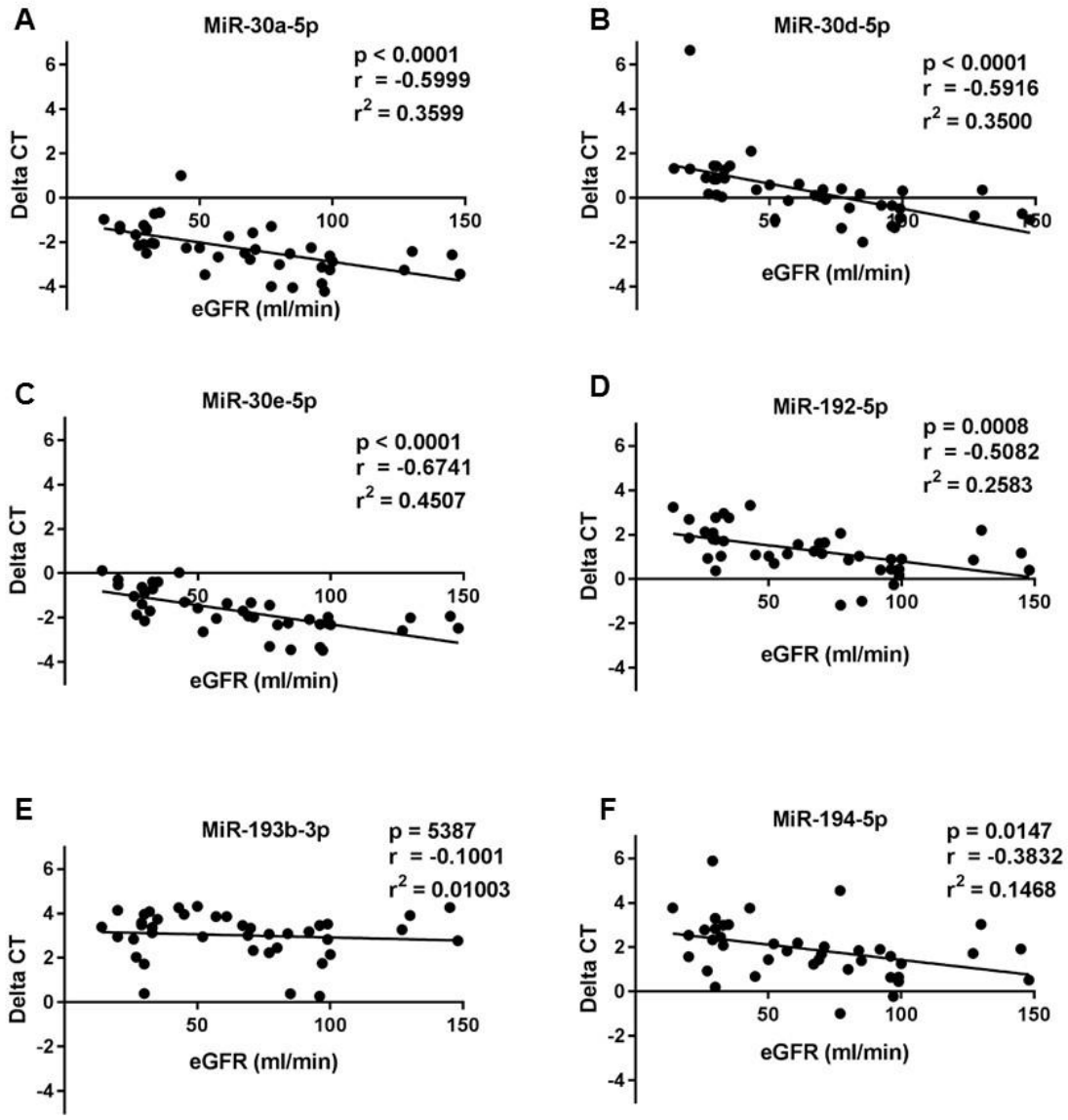

Figure 4.14. Association between differentially expressed microRNAs and eGFR

The figure shows the correlation between (**A**) miR-30a-5p, (**B**) miR-30d-5p, (**C**) miR-30e-5p, (**D**) miR-192-5p, (**E**) miR-193b-3p and (**F**) miR-194-5p expressions in urine exosomes and eGFR for patients with ADPKD as determined by Spearman correlation coefficient.

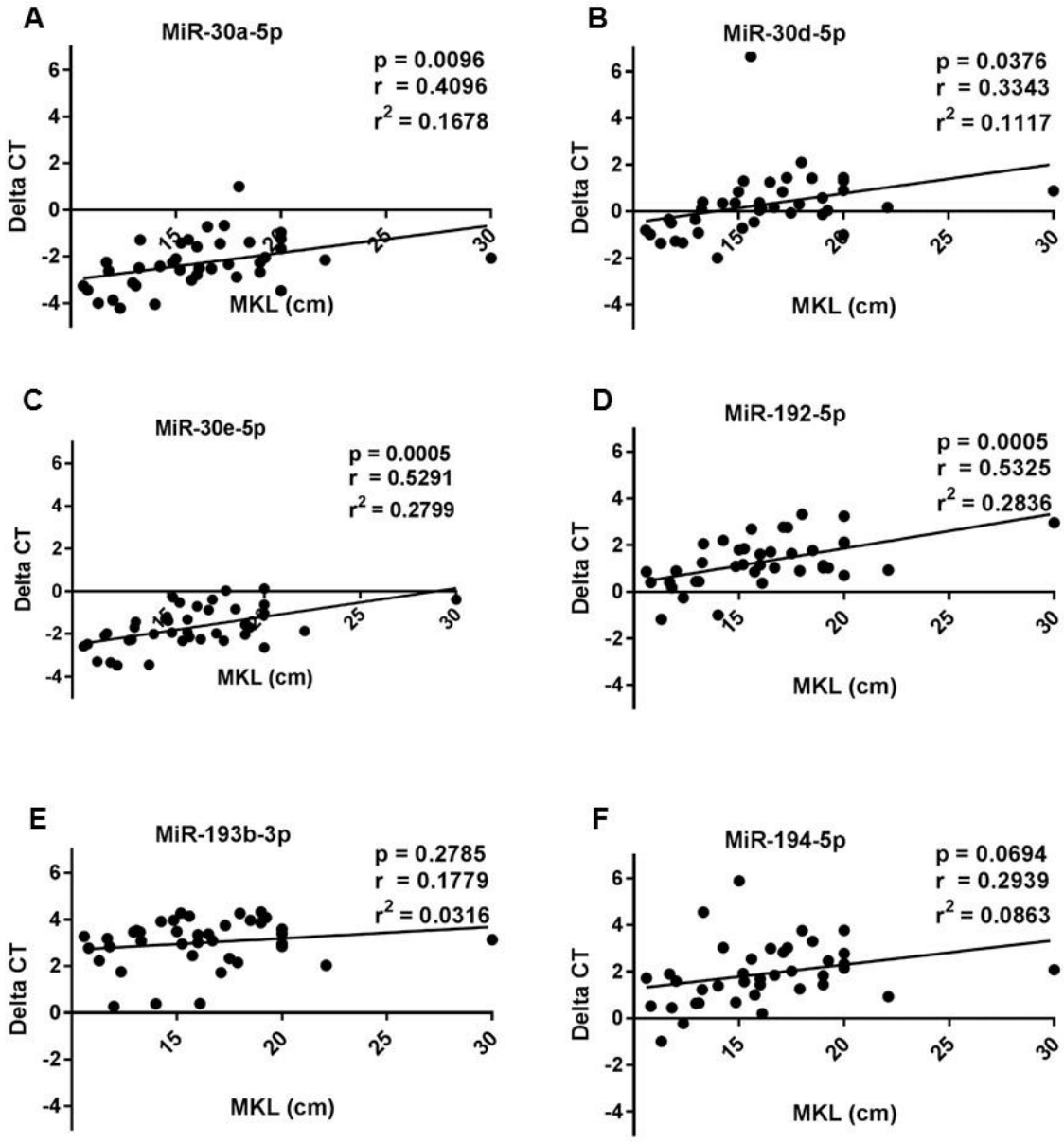

Figure 4.15. Association between differentially expressed microRNAs and MKL

The figure shows the correlation between (**A**) miR-30a-5p, (B) miR-30d-5p, (**C**) miR-30e-5p, (**D**) miR-192-5p, (**E**) miR-193b-3p and (**F**) miR-194-5p expressions in urine exosomes and MKL for patients with ADPKD as determined by Spearman correlation coefficient.

4.8.5 Univariate and multivariate regression analysis

A linear regression analysis was carried out to identify the correlation between the differentially expressed microRNAs and other clinical variables (**Table 4.7**). No univariate correlations were found between the differentially expressed microRNAs and the mean age, BMI and cholesterol. MiR-30a-5p, miR-30e-5p and miR-192-5p showed significant correlations with all structural and functional measurement of renal function including eGFR, eGFR slope and mean kidney length. Additionally, miR-30e-5p and miR-192-5p showed significant correlations with PCR. MiR-30d-5p showed significant correlations with eGFR as well as serum uric acid, whereas, miR-193b-3p showed a significant correlation with eGFR slope. On the other hand, miR-194-5p did not show significant associations with any examined clinical and biochemical variables.

All the clinical and biochemical parameters of patients with ADPKD that revealed a significance univariate association with the examined microRNAs were included in a multiple regression analysis model (**Table 4.8**). Estimated GFR remained significantly associated with the miR-30-family and lost its association with miR-192-5p. MKL remained significantly associated with miR-192-5p and lost its association with miR-30a-5p and 30e-5p. MiR-30d-5p was significantly associated with uric acid. Only miR-193b-3p was significantly associated with eGFR slope.

Table 4.7. Univariate linear regression of differentially expressed microRNAs with clinical and biochemistry variables of ADPKD patients

Variables	MiR-30a-5p Standardized β (P value)	MiR-30d-5p Standardized β (P value)	MiR-30e-5p Standardized β (P value)	MiR-192-5p Standardized β (P value)	MiR-193b-3p Standardized β (P value)	MiR-194-5p Standardized β (P value)
Age (year)	0.128 (0.338)	0.133 (0.4)	0.133 (0.318)	0.091 (0.498)	-0.55 (0.679)	0.164 (0.218)
BMI (kg/m^2)	-0.124 (0.37)	-0.133 (0.418)	-0.065 (0.63)	0.061 (0.907)	0.025 (0.858)	0.031 (0.924)
eGFR (ml/min/1.73m^2)	-0.577 (0.00)	-0.444 (0.002)	-0.550 (0.00)	-0.495 (0.00)	-0.140 (0.359)	-0.288 (0.055)
eGFR slope (ml/min/1.73m^2)	-0.415 (0.01)	-0.275 (0.085)	-0.399 (0.01)	-0.369 (0.01)	-0.418 (0.007)	-0.234 (0.146)
MKL (cm)	0.432 (0.01)	0.232 (0.154)	0.436 (0.006)	0.533 (0.000)	0.178 (0.278)	0.294 (0.069)
Cholesterol (mmol/l)	-0.186 (0.162)	-0.190 (0.15)	-0.222 (0.09)	-0.157 (0.24)	-0.077 (0.564)	-0.066 (0.621)
Uric acid (µmol/l)	0.103 (0.464)	0.466 (0.000)	0.137 (0.328)	0.027 (0.846)	0.249 (0.072)	0.123 (0.328)
PTH (mmol/l)	0.362 (0.036)	0.120 (0.499)	0.301 (0.084)	0.322 (0.064)	-0.195 (0.270)	-0.032 (0.859)
PCR (mg/mmol)	0.358 (0.057)	0.289 (0.128)	0.372 (0.047)	0.458 (0.013)	0.033 (0.866)	0.192 (0.317)

A linear regression analysis for the various clinical and biochemical variables was generated to examine the independent associations between these parameters and the differentially expressed microRNAs. Standardized β (standardized coefficients beta) denotes that an alteration of 1SD in the clinical parameters (independent variable) will lead to a one measurement change in the corresponding differentially expressed microRNAs (dependent factor). P value < 0.05 was considered significant and denotes with red lines.

Table 4.8. Multivariate linear regression of differentially expressed microRNAs with clinical and biochemistry variables of ADPKD patients

Variables	MiR-30a-5p Standardized β (P value)	MiR-30d-5p Standardized β (P value)	MiR-30e-5p Standardized β (P value)	MiR-192-5p Standardized β (P value)	MiR-193b-3p Standardized β (P value)	MiR-194-5p Standardized β (P value)
eGFR (ml/min/1.73m^2)	-0.561 (0.01)	0.317 (0.036)	-0.386 (0.04)	-0.290 (0.12)		
eGFR slope (ml/min/1.73m^2)	-0.215 (0.16)	/	-0.214 (0.16)	-0.236 (0.12)	-0.418 (0.01)	
MKL (cm)	0.075 (0.655)	/	0.134 (0.442)	0.533 (0.000)		
Uric acid (µmol/l)		0.394 (0.010)				
PTH (mmol/l)	-0.035 (0.842)	/				
PCR (mg/mmol)		/				

The table shows a multivariate regression model adjusted to the variables which have significant correlations with the corresponding differentially expressed microRNAs. P value < 0.05 was considered significant and denotes with red lines.

4.8.6 Prediction of ADPKD progression

The ability of the differentially expressed microRNAs to predict rapidly progressive disease (eGFR slope over 5 years) was calculated using ROC curve analysis (SPSS). A loss of renal function > 3 ml/min/1.73m^2 was considered to indicate progressive disease (Rosansky and Glassock 2014). A ROC curve calculation relies on the fraction of the number of true positive (study sensitivity) and the number of false positives (1 - sensitivity).

The ROC curves for individual microRNA are depicted in **Figure 4.16 A** to **F**. In addition, the ROC curve of MKL, combined microRNAs and combinations of microRNAs with MKL were calculated (**Figure 4.16 G** - **I**). The area under the curve (AUC), confidence interval (CI), and the P values of all calculated ROC curves are displayed in **Table 4.9**. The cut-off values of the differentially expressed microRNAs, corresponding sensitivity and specificity, are also displayed in **Table 4.9.**

MiR-193b-3p showed the highest AUC (0.836) with a 72.7% sensitivity and 100% specificity at a cut-off value of 3.38 delta CT, followed by MiR-30e-5p (0.826), whereas, MKL showed the lowest predictive ability (0.634) (**Figure 4.16**). Interestingly, a combination of differentially expressed microRNAs and improved the ability to discriminate patients with a slowly progressive disease from those with a rapidly progressive disease with an AUC of 0.914.

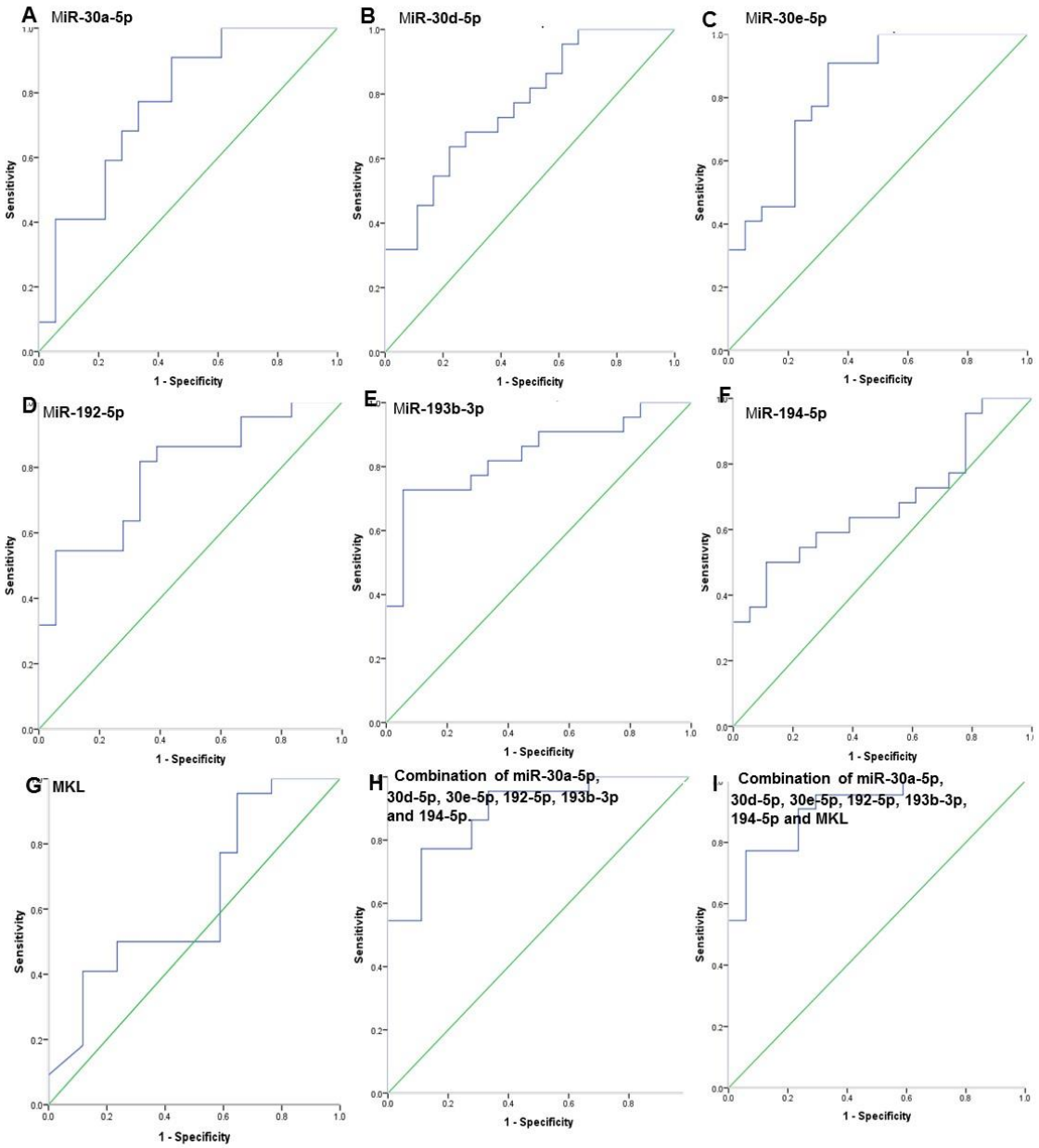

Figure 4.16. Diagnostic performance of differentially expressed microRNAs to predict ADPKD progression

Roc curve for (**A**) miR-30a-5p, (**B**) miR-30d-5p, (**C**) miR-30e-5p, (**D**) miR-192-5p, (**E**) miR-193b-3p, (**F**) miR-194-5p, (**G**) MKL, (**H**) combination of differentially expressed microRNAs and (**I**) combination of the differentially expressed microRNAs and MKL for prediction of eGFR slope. Combination of microRNAs and MKL showed the highest AUC which reflecting the ability to discriminate between patients with risk of CKD rapidly progression from patients with slowly progressive risk. AUC and P value are shown in the table 4.9. Abbreviations are: MKL; Mean Kidney Length, ROC curve; Receiver operating Curve.

Table 4.9. Diagnostic performance of the differentially expressed microRNAs to predict ADPKD progression

Variables	AUC	95% CI	P value	Cut-off value (delta CT)	Sensitivity	Specificity
MiR-30a-5p	0.770	0.620-0.920	0.004	-2.49	77.3%	70.6%
MiR-30d-5p	0.765	0.620-0.911	0.004	0.088	72.7%	64.7%
MiR-30e-5p	0.826	0.695-0.956	0.000	-1.89	72.7%	82.4%
MiR-192-5p	0.785	0.644-0.926	0.002	1.03	81.8%	70.6%
MiR-193b-3p	0.836	0.709-0.963	0.000	3.38	72.7%	100%
MiR-194-5p	0.677	0.510-0.844	0.057	1.42	72.7%	41.2%
MKL	0.634	0.453-0.815	0.157	14.55 cm	72.7%	41.2%
Combination of microRNAs	0.889	0.790-0.988	0.000	/	/	/
Combination of microRNAs and MKL	0.914	0.820-1	0.000	/	/	/

The results of ROC curves for the differentially expressed microRNAs for dichotomized eGFR slope are shown in the table. A perfect diagnostic test has AUC of 1 while a value of 0.5 indicates weak prediction ability. 95 % CI indicates 95 confidences of the true AUC of the population. Red lines represent statistically significant P values, < 0.05. The cut-off value for each differentially expressed microRNAs, beside mean kidney length, were calculated based on the value that provided maximum sensitivity and specificity for ADPKD progression. Abbreviations are: ROC curve; Receiver operating Curve, AUC; Area under the curve, CI; Confidence Interval, P value; probability value, MKL; Mean Kidney Length.

4.9 MicroRNAs target prediction and pathways enrichment

Following the validation of differentially expressed individual microRNAs in urinary exosomes, it was of interest to study the cellular pathways targeted by these microRNAs to determine the potential functional significance of their downregulation in ADPKD.

Predicted target genes of validated microRNAs were identified using the miRWalk database (version 2.0) (Dweep, Sticht et al. 2011; Dweep and Gretz 2015). MiRWalk identifies target genes based on a comparison of 5 separate prediction algorithms, namely, targetscan, miRanda, miRWalk, RNA22 and miRDB. Default parameters were used to determine the predicted genes. Genes which were identified by at least 3 different algorithms were included for downstream analysis, since the combination of various prediction algorithms can reduce the risk of false positive target gene prediction (Dweep, Sticht et al. 2013).

The list of differentially expressed microRNA target genes was then analysed by a DAVID pathway online analysis program to identify functional pathway annotations of these genes (The **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery, DAVID, version 6.8) using recommended settings (Huang, Sherman et al. 2007; Huang da, Sherman et al. 2009; Godard and van Eyll 2015), this algorithm is available at https://david.ncifcrf.gov/. Fisher's exact test was used to identify target pathways, and pathways with p value < 0.05 were considered as enriched pathways (Manoli, Gretz et al. 2006; Pandey, Brors et al. 2008). A total of 11 significantly enriched target pathways were identified, and summarized in **Table 4.10**.

Table 4.10. MicroRNAs enriched pathways

Significantly enriched pathways	MiR-30a-5p P value (fold enrichment)	MiR-30d-5p P value (fold enrichment)	MR-30e-5p P value (fold enrichment)	MiR-192-5p P value (fold enrichment)	MiR-193b-3p P value (fold enrichment)	miR-194-5p P value (fold enrichment)
Wnt signaling	0.02 (1.5)	0.01 (1.5)	0.02(1.5)	ns	0.011 (1.7)	4.45E-04 (2)
MAPK signaling	0.02 (1.3)	0.03 (1.3)	0.01 (1.4)	0.04 (1.5)	/	ns
ErbB signaling	0.02 (1.7)	0.01 (1.8)	0.007 (1.8)	ns	0.006 (2.2)	ns
Calcium signaling	0.04 (1.4)	0.04 (1.4)	0.03 (1.4)	ns	0.04 (1.5)	ns
Apoptosis	0.003(1.98)	0.006 (1.9)	0.01 (1.7)	0.01 (2.3)	ns	ns
P53 signaling	ns	ns	0.03 (1.7)	ns	0.001 (2.6)	ns
Cell cycle	ns	ns	ns	0.03 (1.9)	ns	ns
Endocytosis	ns	ns	ns	ns	ns	0.002 (1.7)
Focal adhesion	ns	ns	ns	ns	ns	0.01 (1.5)
JAK-STAT signaling	ns	ns	ns	ns	ns	0.02 (1.6)
mTOR signal	ns	ns	ns	ns	ns	0.01 (2.3)

The table shows the significantly enriched cellular pathways based on the predicted target genes of differentially expressed microRNAs as identified by DAVID pathway analysis. Abbreviation is: ns; non-significant.

4.10 Gene predicted targets of differentially expressed microRNAs and their values in ADPKD

Among the differentially expressed microRNAs target pathways, I selected the top 3 pathways, Wnt, MAPK and ErbB signalling pathways, to detect validated target genes and to examine the expressions of target genes in urine exosomes of patients with ADPKD. Wnt signalling was selected because it was the top targeted signalling pathways by miR-30a-5p, miR-30d-5p, miR-30e-5p, miR-193b-3p and miR-194-5p (**Figure 4.17**). Wnt signalling has 2 major branches i.e. β-catenin-mediated (canonical) and non-canonical signalling pathways, which share some common components (Saigusa and Bell 2015; Kim, Nie et al. 2016). The canonical signalling regulates β-catenin stabilization, cell proliferation and apical-basal membrane polarity (Benzing, Simons et al. 2007; McNeill 2009; Saigusa and Bell 2015; Kim, Nie et al. 2016). Non-canonical signalling involves Wnt/calcium and planer cell polarity (PCP) pathways (Benzing, Simons et al. 2007). Both canonical and non-canonical pathways may play roles in cystogenesis (Benzing, Simons et al. 2007; Wuebken and Schmidt-Ott 2011; Goggolidou 2014). Wnt signalling is essential for various biological functions including cell proliferation, polarity, migration and organ development (Saigusa and Bell 2015). In this regard, recent work reporting that the extracellular domain of PC1 can act as a Wnt co-receptor involved in WNT/Ca^2 signalling supports a direct role for Wnt in the pathogenesis of ADPKD (Kim, Nie et al. 2016).

MiR-30 family and miR-192-5p were predicted to target MAPK pathway through the targeting of ligands, receptors and transcription molecules of this pathway (**Figure 4.18**). The importance of MAPK signalling pathway in the pathogenesis of ADPKD has been reported many times (Torres and Harris 2006; Saigusa and Bell 2015). MAPK signalling participates in many cellular process involved in cystogenesis including proliferation, differentiation, phosphorylation and apoptosis (Sorenson and Sheibani 2002; Nagao, Yamaguchi et al. 2003; Wada and Penninger 2004), and activation of MAPK pathways has been documented in cystic tissue of (Cy/+) heterozygous rats (Nagao, Yamaguchi et al. 2003) and bcl-2 -/- mice (Sorenson and Sheibani 2002).

Finally, miR-30 family and miR-193b-3p were predicted to target the ErbB pathway through regulation of its various molecules (**Figure 4.19**). A recent study from our laboratory has found overexpression of ErbB4 in cystic kidney of human and animal models of ADPKD as well as in urine exosomes from patients with ADPKD (Streets, Magayr et al. 2017). Therefore, I decided to examine ErbB4 gene expression in urine exosomes from patients with ADPKD.

From these pathways, I chose to focus on genes targeted by 2 or more of the differential expressed microRNAs. Based on these criteria, I selected the following molecules: (1) WNT pathway: a ligand (WNT5a), a receptor (FZD3) and a transcription factor (NFAT5); (2) MAPK pathway: 2 receptor ligands, FGF-2 and FGF5 (3) ErbB pathway: ErbB4. FZD4 as a positive control since it was reported to be a predictor of disease progression in patients with ADPKD (Romaker, Puetz et al. 2009; Zschiedrich, Budde et al. 2016). The clinical parameters of the study population are described in **Table 4.5**.

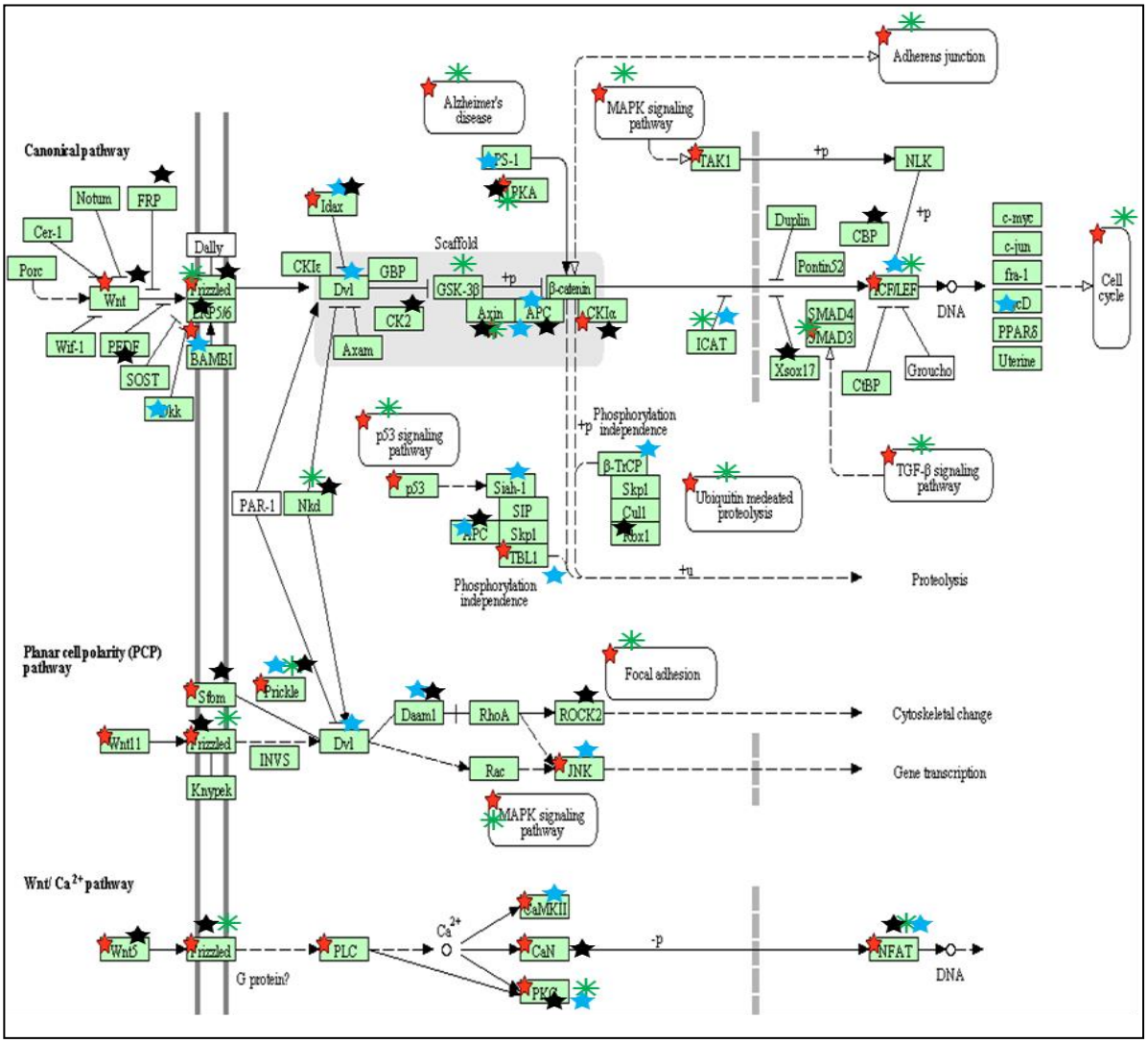

Figure 4.17. Wnt signalling pathway

The figure shows a schematic representation of various components of Wnt pathway as targets of differentially expressed microRNAs. Every microRNA is represented on the graph with a different symbol: miR30 family = ★, miR192 = ✳, miR-193b-3p = ★ and miR194 = ★.

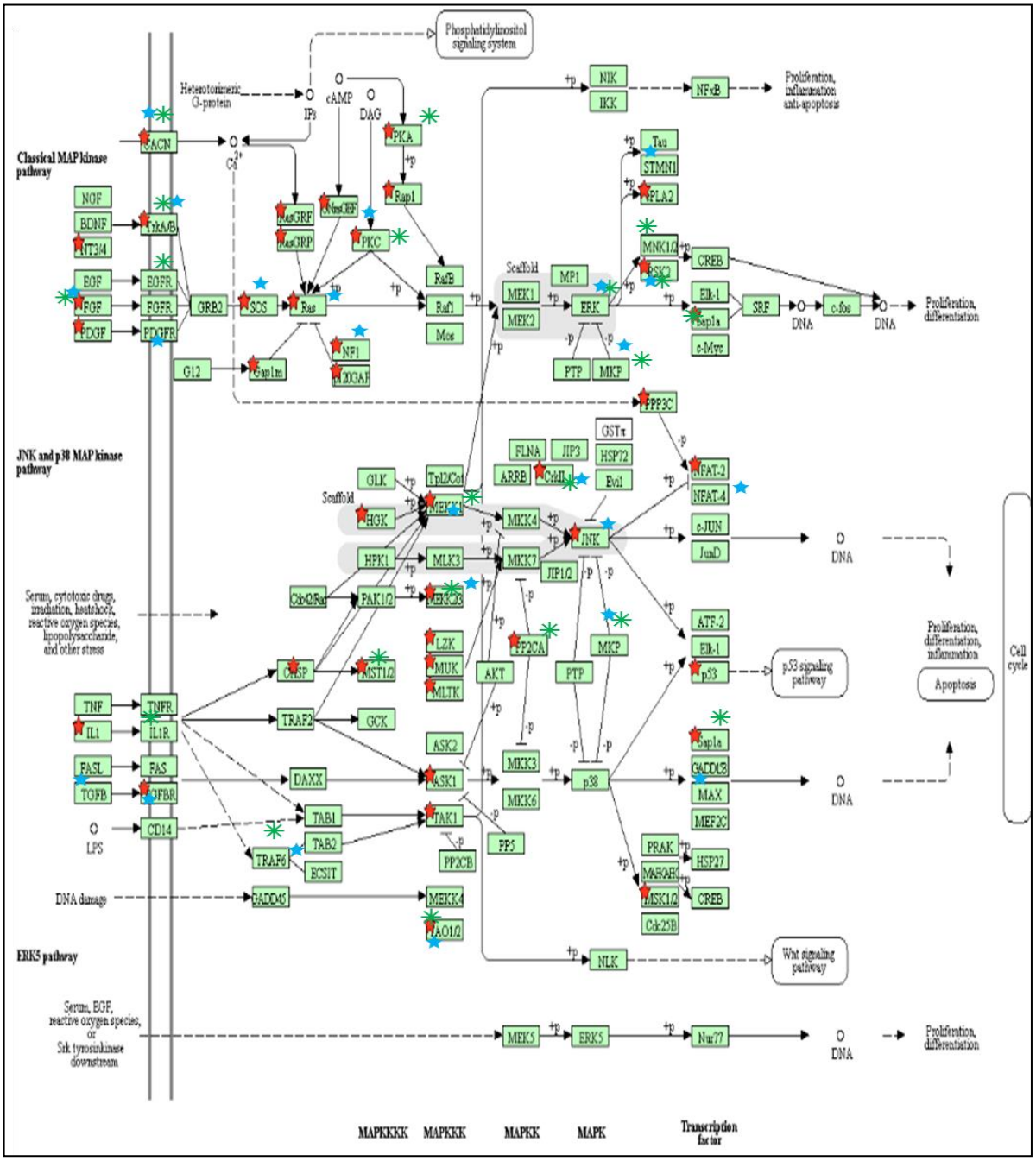

Figure 4.18. Mitogen-activated protein kinase (MAPK) signalling pathway

The figure shows a schematic representation of various components of MAPK pathway as targets of differentially expressed microRNAs. Every microRNA is represented on the graph with a different symbol: miR30 family = ★, miR192 = ✳ and miR-193b-3p = ★.

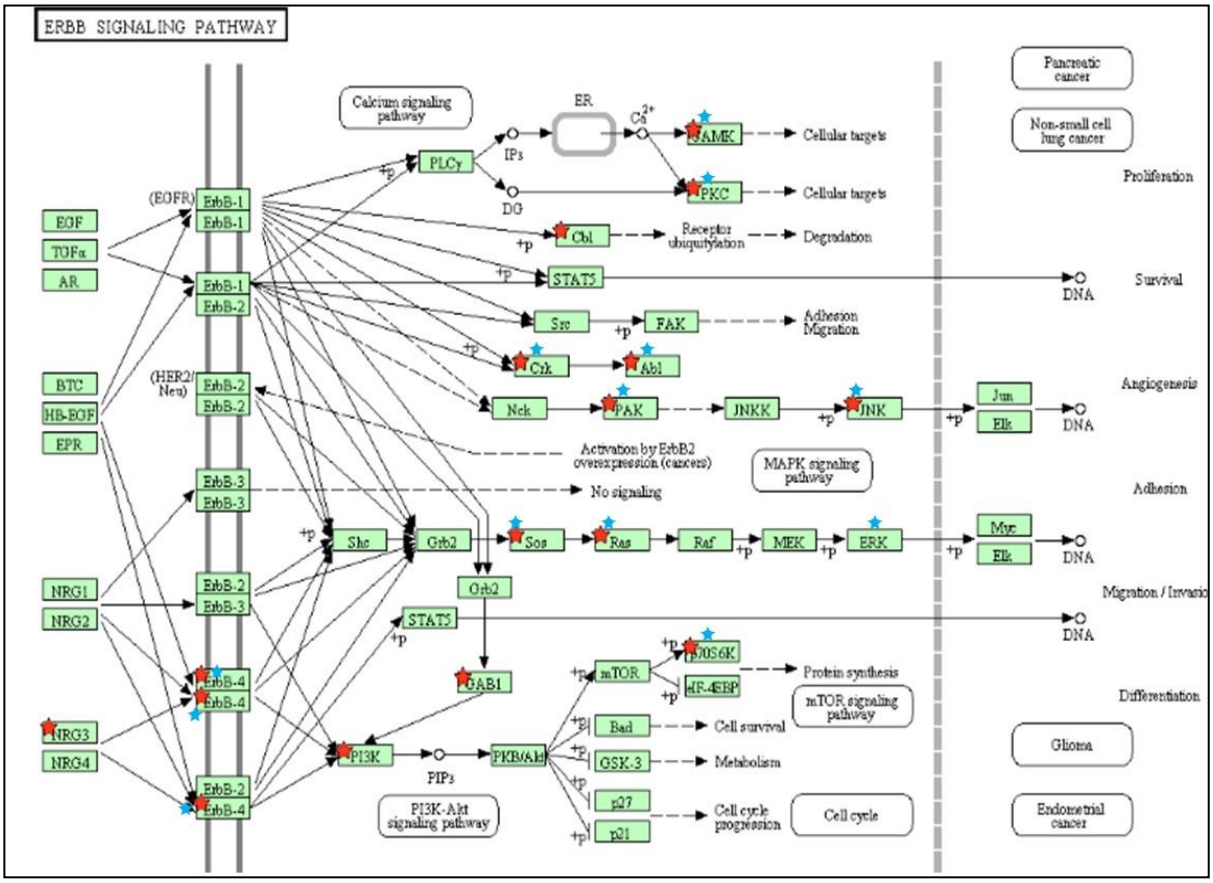

Figure 4.19. ErbB signalling pathway

The figure shows a schematic representation of various components of ErbB pathway as targets of differentially expressed microRNAs. Every microRNA is represented on the graph with a different symbol: miR30 family = ★ and miR-193b-3p = ★.

4.11 Analysis of urinary mRNAs targeted by differentially expressed microRNAs in patients with ADPKD and healthy controls

Initially, the expression levels of the selected mRNA were compared in patients with ADPKD (n = 40) and healthy controls (n = 20) using TaqMan gene expression qPCR assays. GAPDH mRNA was selected as a normalising housekeeping gene based on previous exosome associated mRNA studies (Lv, Cao et al. 2014; Sole, Cortes-Hernandez et al. 2015). GAPDH mRNA expression was not significantly different between urine samples from healthy controls, ADPKD patients with eGFR > 60 ml/min and ADPKD patients with eGFR < 60 ml/min (**Figure 4.20**).

All examined molecules showed high CT values (~34) indicating low abundance. **Figure 4.21 A** – **G** shows the expression levels of individual mRNA in the patients and healthy controls. Only FGF5 expression was significantly higher in the urine exosomes from patients with ADPKD when compared to healthy controls (fold change 1.53) (**Figure 4.21F**).

The expression of FGF5 was compared between ADPKD patients with an eGFR> 60 ml/min (n = 20), those with eGFR < 60 ml/min (n = 20) and healthy controls (n = 20) using one-way ANOVA, and summarized in **Figure 4.22A**. FGF5 expression in urine exosomes was significantly higher in the samples from patients with eGFR < 60 ml/min as compared to healthy controls. No significant difference was however found between patients with eGFR > 60 ml/min and the patients with eGFR < 60 ml/min in the FGF5 expressions in urine exosomes.

Furthermore, the expression of FGF5 in urine samples from the patients with eGFR slope > 3 ml/min (n = 22), the patients with eGFR slope < 3 ml/min (n = 17) and healthy controls (n = 20) were compared using one-way ANOVA followed by a Tukey's post hoc analysis. FGF5 expression was higher in urine samples from the patients with eGFR > 3 ml/min (fold change 1.94) when compared to healthy controls. There was no significant difference in its expressions in urine samples from the patients with eGFR > 3 ml/min when compared to the patients with eGFR < 3 ml/min (**Figure 4.22B**).

The associations of FGF5 with eGFR and MKL were also determined in this study by Spearman-correlation test. No significant correlations were found between FGF5 expression in urine exosomes from ADPKD patients with eGFR or MKL. In addition, the utility of FGF5 in predicting disease progression in ADPKD was calculated based on an eGFR slope using a ROC curve (SPSS). The results showed that the ability of FGF5 to predict ADPKD progression was not better than by chance.

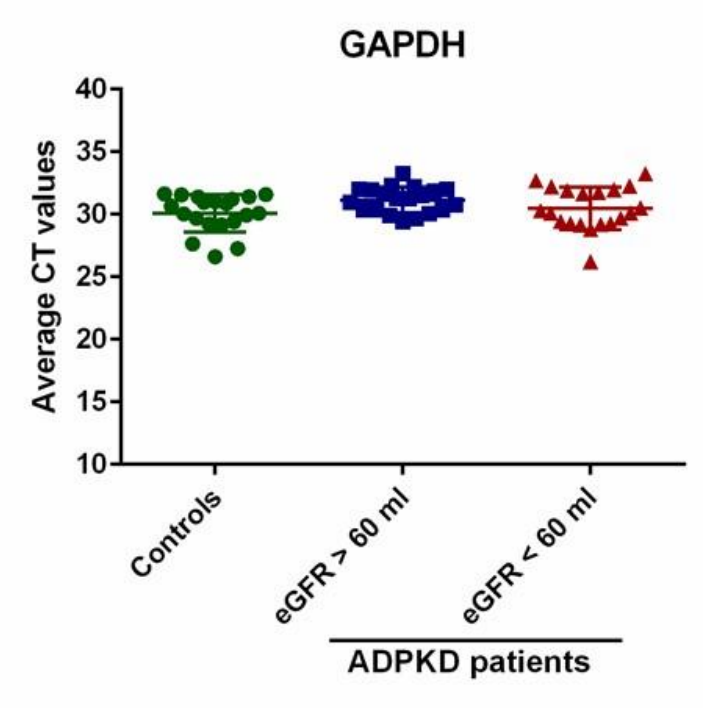

Figure 4.20. Expression of GAPDH mRNA in urinary exosomes

The figure shows the expression of GAPDH mRNA (an endogenous control) in urine exosomes from healthy controls (n = 20), ADPKD patients with eGFR > 60 ml/min (n = 20) and ADPKD patients with eGFR < 60 ml/min (n = 20).

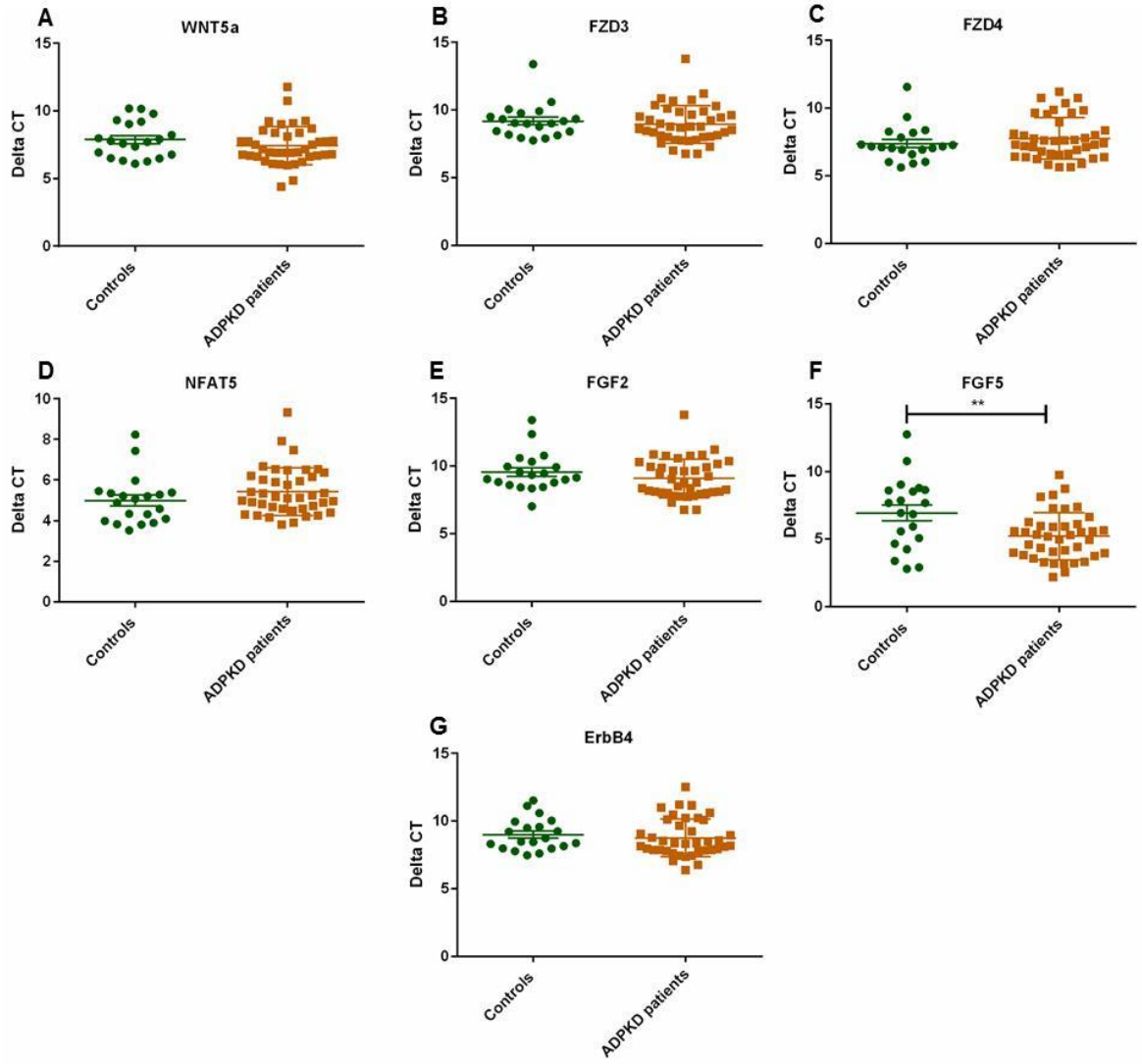

Figure 4.21. Urinary mRNA expressions in urine exosomes

The figure shows the expressions of (**A**) WNT5, (**B**) FZD3, (**C**) FZD4, (**D**) NFAT5, (**E**) FGF2, (**F**) FGF5 and (**G**) ErbB4 in urine exosomes as determined by TaqMan qPCR assays in healthy controls (n = 20) and patients with ADPKD (n = 40). Corrected results to GAPDH, are display in the graph. P value > 0.05 was considered non-significant, * denotes P value < 0.05; ** denotes P value < 0.01; *** denotes P value < 0.001 and **** denotes P value < 0.0001. Data is displayed as mean ± SEM.

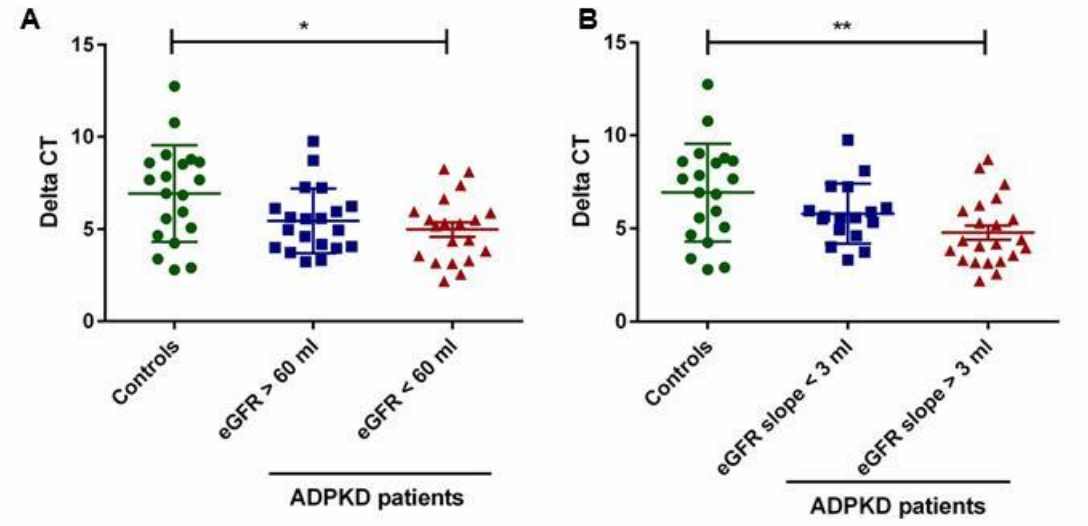

Figure 4.22. Urinary FGF5 expression in urine exosomes

(**A**) The figure shows the relative expression levels FGF5 in urine exosomes as determined by qPCR in healthy controls (n = 20), patients with eGFR > 60 ml/min (n = 20) and patients with eGFR < 60 ml/min (n = 20).

(**B**) The figure shows the relative expression levels FGF5 in urine exosomes as determined by qPCR in healthy controls (n = 20), patients with eGFR slope < 3 ml/min (n = 17) and patients with eGFR slope > 3 ml/min (n = 22).

Corrected results, to GAPDH, are display in the graph. One-way ANOVA test was used to analysis the data. P value > 0.05 was considered non-significant, * denotes P value < 0.05; ** denotes P value < 0.01; *** denotes P value < 0.001 and **** denotes P value < 0.0001. Data is displayed as mean ± SEM.

4.12 Summary

The key findings in this chapter were:

1. MicroRNAs were expressed at very low levels in cell free urine.

2. Exosomes were enriched for microRNAs compared to cell free urine.

3. All differentially expressed microRNAs were downregulated in urine samples from patients with ADPKD as compared to the controls, and these results were confirmed in a separate cohort by qPCR.

4. Differentially expressed microRNAs in urine were negatively correlated with kidney function tests (eGFR), and positively correlated with MKL.

5. Differentially expressed microRNAs were significantly better predictors of the rate of disease progression in ADPKD compared to mean kidney length and together had a combinatory effect.

6. Differentially expressed microRNAs were predicted to target a number of genes which participate in WNT signalling, the MAPK and ErbB pathways.

4.13 Discussion

MicroRNAs play a critical role in modulating gene expression in health and disease (Jones 2007; Santos-Reboucas and Pimentel 2007; Asirvatham, Magner et al. 2009). A single microRNA can regulate the function of hundred genes (Asirvatham, Magner et al. 2009). Interestingly, a number of genes involved in cystic kidney are regulated by microRNAs including *PKD1, PKD2,* bicaudal C (Bicc1) and hepatocyte nuclear factor-1β (Tran, Zakin et al. 2010; Patel, Hajarnis et al. 2012; Patel, Williams et al. 2013). The potential role of microRNAs in the pathogenesis of cystic disease has been reported (Sun, Li et al. 2010; Patel, Williams et al. 2013; Lakhia, Hajarnis et al. 2016), but no study to date has evaluated the utility of urinary microRNAs as potential prognostic biomarkers in ADPKD.

In this project, I initially used qPCR panel arrays (Exiqon) to screen a limited number (752) of microRNAs in cell free urine of patients with ADPKD. This pilot study identified a panel of 7 dysregulated microRNAs in ADPKD compared to diabetic, hypertensive patients and healthy controls. However, the very low abundance of microRNAs in cell free urine made consistent results difficult to obtain probably due to variable extraction efficiency and therefore made further validation impossible. These results are consistent with those from another group which found similar low quantity and quality of microRNAs in cell free urine (Saikumar, Hoffmann et al. 2012; Nassirpour, Mathur et al. 2014). However, other groups have reported the usefulness of the cell free microRNAs as a diagnostic biomarker (Zhang, Lau et al. 2014; Korzeniewski, Tosev et al. 2015). The discrepancy between our results and these studies could be due to several reasons such as the lack of an accepted standardized protocol for sample processing, optimal microRNA extraction technique, and the lack of an accepted normalization approach (Nakamura, Sawada et al. 2016).

Barcode next generation sequencing was therefore applied to profile exosome associated microRNAs in ADPKD patients with eGFR > 60 ml/min, ADPKD patients with eGFR < 60 ml/min and healthy controls. This approach identified several differentially expressed microRNAs. A consistent finding was that all differentially expressed microRNAs (including miR-30 family, miR-192-5p, miR-194-5p and miR-193b-3p) showed significantly reduced expression in patients with advanced disease (eGFR < 60 ml/min) or those with rapid disease progression (eGFR slope > 3 ml/min over 5 years) when compared to the patients with early disease, slow disease progression or healthy controls. Only miR-192-5p expression was significantly decreased in patients with eGFR > 60 ml/min when compared to healthy controls. In a multivariate analysis, all the differentially expressed microRNAs (except miR-194-5p)

showed significant correlations with eGFR slope. In addition, significant correlations were found between miR-30 family and miR-192-5p with eGFR but only miR-30a-5p, miR-30e-5p and miR-192-5p showed significant correlations with MKL. Based on calculation of ROC curves, all differentially expressed microRNAs showed a significant ability to predict ADPKD progression outperforming MKL. These results suggest that these microRNAs play important and non-redundant roles in different stages of cystogenesis.

A trivial explanation for the lower expression of microRNAs in ADPKD urine samples could be the secretion of less exosomes in disease. However, similar levels of exosome excretion has been shown in healthy controls and ADPKD patients at different CKD stages (Pocsfalvi, Raj et al. 2015). In addition, DESeq normalization for next generation sequencing reads was used by different software. Therefore, the down regulation of the differentially expressed microRNAs cannot be contributed to the lower exosome content or to lack of a normalization method. Similar to my results, all detected microRNAs were downregulated in cancer tissues compared to the controls (Jansson and Lund 2012; van Schooneveld, Wildiers et al. 2015). The alteration in microRNAs expressions in cancer is likely either to alteration of microRNAs genes by amplification or deletion, deregulation of transcriptional control of microRNAs such as P53, defects in any components of microRNAs biogenesis such as Dicer or dysregulation of other epigenetic factors for instance hypo or hyper-methylation of DNA (Peng and Croce 2016).

Whether these microRNAs are associated with specific disease processes related to cystogenesis or instead represent non-specific markers associated to common pathways related to renal failure is not clear from this study. In one previous study, the expression of microRNAs present in urine cellular sediment and exosomes in ADPKD (n = 20) and CKD (n = 20) patients was compared to those present in primary cell cultures derived from cystic and normal kidney (Ben-Dov, Tan et al. 2014). The authors identified 8 microRNAs significantly downregulated in ADPKD exosomes compared to CKD exosomes including miR-133a, miR-1, miR-671, miR-378a, miR-221-5p and miR-98. Interestingly, in our results, miR-378a and miR-221-5p were also significantly reduced in ADPKD patients when compared to healthy controls.

Of the microRNAs altered, a striking observation was the down regulation of 3 members of the miR-30 family (miR-30a-5p, 30d-5p and 30e-5p) in ADPKD exosomes especially given the links this microRNA family has to a number of signalling pathways previously implicated in cystogenesis. The miR-30 family plays a significant role in the regulation of calcium/calcineurin signalling in renal podocytes (Wu, Zheng et al. 2015)

and has also been linked to apoptosis and proliferation (Dai, Kang et al. 2014; Ma, Li et al. 2016). The miR-30 family regulates apoptosis through direct stimulation of p53 in cardio myocytes or through targeting mitochondrial fission (Li, Donath et al. 2010). It is known that p53 is a suppressor for the *PKD1* promotor (van Bodegom, Roessingh et al. 2010). Interestingly, downregulation of the miR-30 family also promotes EMT (epithelial-mesenchymal transition) (Joglekar, Patil et al. 2009; Braun, Hoang-Vu et al. 2010; Togawa, Nakanishi et al. 2011). The miR-30 family has been linked to Wnt signalling (Zhao, Lin et al. 2014). Shi et al. showed that downregulation of miR-30 family by SMAD2 is required for apoptosis induction by TGF-β in podocytes (Shi, Yu et al. 2013). It is well established that TGF-β1/Smad signalling plays a crucial role in the pathogenesis of renal fibrosis (Yao, Yang et al. 2015). Interestingly, there is evidence that TGF-β signalling is augmented in cystic epithelial cells (Song, Di Giovanni et al. 2009; Liu, Wang et al. 2014; Weng, Wang et al. 2015).

Intriguingly, miR-192-5p and miR-194-5p were also among the differentially altered microRNAs in my study. MiR-192 is known to exert a negative feedback effect on TGF-beta/Smad3-signalling in renal fibrosis and miR-192 expression found to be inversely correlated with the degree of interstitial fibrosis in proximal tubular cells (Krupa, Jenkins et al. 2010; Park, Woo et al. 2011). It is interesting to note that miR-192 is preferentially expressed in renal cortex (~20 fold) compared to medulla raising a possible role in the regulation of sodium transport; alteration in the sodium and water transport in ADPKD has been reported (D'Angelo, Mioni et al. 1975; Avner, Sweeney et al. 1992; Tian, Greene et al. 2008; Liang, Liu et al. 2009). MiR-192 and miR-194 are under the control of p53 and the role of p53 in ADPKD pathogenesis has been previously established (Nishio, Hatano et al. 2005; Van Bodegom, Saifudeen et al. 2006; Pichiorri, Suh et al. 2016). Additionally, these 2 microRNAs have a putative binding site on Ets-1, which is a transcription factor implicated in ADPKD pathogenesis (Sun, Koo et al. 2004; Puri, Rodova et al. 2006).

The final differentially expressed microRNA in this study was miR-193b-3p. This microRNA was recently identified by our group independently from parallel microarray profiling of cystic epithelial cell lines (Streets, Magayr et al. 2017). MiR-193b-3p expression in urine exosomes was significantly lower in ADPKD urine compared to healthy controls. ROC curve as well as multivariate analysis showed that miR-193b-3p maybe an independent prognostic biomarker for disease progression in ADPKD. We have recently shown that miR-193b-3p can bind to the 3'UTR of the EGF/ErbB receptor ErbB4 (Streets, Magayr et al. 2017). In addition, miR-193b-3p is known to regulate cyclin D1 in various cancer tissues including prostate and multiple myeloma (Chen,

Feilotter et al. 2010). Increased cyclin D1 and P-Rb have been associated with early cyst growth in rat and human cystic kidneys (Chen, Feilotter et al. 2010; Kaukoniemi, Rauhala et al. 2015). Increase expression levels of miR-193b have been reported as a tumour suppressor through inhibition of cell proliferation and migrations, whereas, decrease its expression levels have been implicated in the progression of a number of malignance including gastric cancers (Hu, Li et al. 2012; Gastaldi, Bertero et al. 2014; Yang, He et al. 2014; Jin, Sun et al. 2015; Mitra, Chiang et al. 2015; Guo, Luo et al. 2016). MiR-193b was reported to be associated with MAPK signal activity, TGF-β signalling, receptor tyrosine kinases signal, which are implicated in the pathogenies of ADPKD (Ikeda, Tanji et al. 2012; Haetscher, Feuermann et al. 2015; Hou, Yang et al. 2015; Zhou, Li et al. 2016). In addition, previous reports has shown that gene encoding urokinase-type plasminogen (uPA) (a serine proteases plasminogen activators) is a direct target of miR-193b, and the role of uPA in renal fibrosis is well established (Zhang, Kim et al. 2003; Li, Yan et al. 2009; Norman 2011; Li, Kong et al. 2014; Iwamoto, Vettori et al. 2016). Of interest, miR-193b-3p expression level might represents an independent predictor factors for disease progression in patients with ovarian cancer (Li, Xu et al. 2015) or those with a history of radical nephrectomy for renal cell carcinoma (Trevisani, Ghidini et al. 2016).

The final section of this chapter was concerned with microRNAs target gene expression. It is well known that microRNAs negatively control gene expression through stimulate mRNA degradation or by inhibiting translation (Bartel 2004; Olena and Patton 2010). So far, there have been very few papers reporting the expression of mRNAs in urine exosomes (Lv, Cao et al. 2014; Sole, Cortes-Hernandez et al. 2015). For instance, one study found lower expression of CD2AP mRNA in urine samples from CKD patients (n = 32) when compared to controls (n = 7), and also a negative correlation between CD2AP expression with proteinuria and tubule-interstitial fibrosis. Similarly, Cortes et al. found a positive corelation between SMAD3 and the chronicity index of lupus nephritis, as well as a negative correlation with miR-29-c expressions.

According to bioinformatics analysis, the differentially expressed microRNAs were predicted target of several common molecular signalling which are implicated in the pathogenesis of cystic kidney diseases including the Wnt/β-catenin signalling pathway (Benzing, Simons et al. 2007), mitogen-activated protein kinase (MAPK) (Torres and Harris 2006; Kim and Choi 2010); ErbB signalling (Torres and Harris 2006); calcium signaling (Calvet 2015); apoptosis (Goilav 2011; Zhou and Li 2015); p53 signaling (van Bodegom, Roessingh et al. 2010); cell cycle (Li, Luo et al. 2005; Chapin and Caplan 2010); endocytosis (Obermuller, Kranzlin et al. 2001); focal adhesion (Drummond

2011); JAK-STAT signaling (Weimbs, Olsan et al. 2013); and mammalian target of rapamycin (mTOR) signal (Mostov 2006). These results suggested that the differentially expressed microRNAs could play functional roles in pathways previously implicated with ADPKD.

To provide experimental validation of this observation, expression of several predicted mRNA targets linked to Wnt, MAPK and ERBB signalling (including Wnt5a, FZD3, FZD4, NFAT5, FGF2, FGF5 and ErbB4) were examined in urine exosomes from 60 participants. Among these molecules, mRNA levels were very low in abundance. Only FGF5 was found to have a significantly higher expression in urine exosomes from patients with late disease (eGFR < 60 ml/min) and those with more rapidly progressive disease (eGFR > 3 ml/min over 5 years). However, FGF5 levels did not show a significant correlation with eGFR or MKL.

The fibroblast growth factor family (FGF) includes 18 members (FGF1-18) and different members have been linked to the pathogenesis of various human diseases (Nguyen, Roux et al. 1988; Beenken and Mohammadi 2009). Some FGF members have been found to be over-expressed in serum and cystic ADPKD models (Nakamura, Ebihara et al. 1993; Grantham 1997; Kuo, Norman et al. 1997; Pavik, Jaeger et al. 2011). Although FGF5 mRNA is expressed at various sites of the mice embryo, its expression in adult mice is normally restricted to the nervous system (Haub, Drucker et al. 1990; Haub and Goldfarb 1991; Allerstorfer, Sonvilla et al. 2008). FGF5 promotes cancer progression through increases in MAPK activity, proliferation, migration and growth of tumour cells (Kornmann, Ishiwata et al. 1997; Allerstorfer, Sonvilla et al. 2008; Fang, Chang et al. 2015). FGF5 plays a crucial role in the regulation of hair growth and length (Hebert, Rosenquist et al. 1994; Higgins, Petukhova et al. 2014). However, to date, no other studies have examined the expression of FGF5 in ADPKD.

Finally, some limitations of this study should be noted. The very low abundance of exosome mRNAs was a major limiting factor. It is possible that altered mRNAs were present in other cellular compartments other than exosomes. Target prediction for microRNA-mRNA pairs relied completely on bioinformatics analysis and was not directly verified with the exception of miR-193b-3p and ErbB4. Finally, it is possible that the primary effect of the microRNAs was mediated through inhibiting mRNA translation (and hence protein levels) rather than through altering mRNA stability (and mRNA levels). Further studies will be required to clarify these points.

Chapter 5 Urinary angiogenesis proteomics array

5.1 Introduction

Although most research in angiogenesis has emphasised its role in tumour growth, other diseases are also strongly linked with an increase in angiogenesis including psoriasis, endometriosis and arthritis (Folkman 1971; Folkman 1995; Folkman 2006). In the context of kidney diseases, imbalances in angiogenic stimulatory and inhibitory factors have been identified as contributing factors to the progression of CKD (Cooper, Vranes et al. 1999; Kang, Anderson et al. 2001; Kang, Joly et al. 2001; Long, Woolf et al. 2001; Maeshima and Makino 2010). Moreover, manipulation of angiogenic factors as a potential therapeutic strategy to slow the rate of CKD progression has been addressed extensively in several studies e.g. inhibition of VEGF successfully improved renal function in mouse models of diabetic nephropathy (de Vriese, Tilton et al. 2001; Flyvbjerg, Dagnaes-Hansen et al. 2002; Schrijvers, De Vriese et al. 2005; Bai, Li et al. 2014).

During the course of ADPKD, kidney volume is massively increased secondary to the growth of a large number of cysts, which ultimately accelerate renal impairment (Sise, Kusaka et al. 2000; Grantham and Calvet 2001). As cysts expand, remodelling of the vasculature occurs in proportion to the degree of cyst expansion (Bello-Reuss, Holubec et al. 2001; Wei, Popov et al. 2006). Radiological corrosion casting-combined with electron microscopy further supports this observation in human polycystic kidneys (Bello-Reuss, Holubec et al. 2001; Wei, Popov et al. 2006). Both studies found a rich network of capillaries surrounding cysts with tuft-like vessels.

In addition, increased concentrations of angiogenic factors have been identified in human ADPKD cystic kidney, renal and hepatic cyst fluid (Bello-Reuss, Holubec et al. 2001; Nichols, Gidey et al. 2004) and mouse ADPKD cholangiocytes (Fabris, Cadamuro et al. 2006). These include VEGF, VEGFR-1, VEGFR-2 and endothelin (Stringer, Komers et al. 2005). Reed (2011) found a positive correlation between serum angiogenic factors with the severity of cardiac and kidney disease and a negative correlation with creatinine clearance in a cohort of 71 young (mean age 16 years) patients with ADPKD (Reed, Masoumi et al. 2011). These studies suggest that changes in angiogenic factors could occur early in polycystic kidneys and reflect the development and growth of renal cysts.

5.2 Hypothesis

Based on the potential role of angiogenesis in cyst growth, I hypothesised that changes in specific urinary angiogenic factors could be detected early in ADPKD and correlate with the rate of renal disease progression.

5.3 Aim

The major aim of this chapter was to identify specific angiogenic factors that could be detected early in disease before permanent damage occurs. Three groups were analysed: patients with early disease (eGFR > 60 ml/min/1.73m^2), those with late disease (eGFR < 60 ml/min/1.73m^2) and healthy controls.

5.4 Objectives

To achieve this aim, the following objectives were performed:

1. To identify differentially expressed angiogenic factors expression in the urine of ADPKD patients compared to controls.

2. To validate changes in selected biomarkers in a larger cohort by ELISA.

3. To correlate the most promising biomarkers with structural and functional measures of renal function and their potential to predict ADPKD progression.

5.5 Discovery profiling of angiogenesis related proteins

The profiles of 55 angiogenesis related proteins were first analysed in pooled urine samples using commercial antibody-based proteomics arrays (R & D Systems, UK).

5.5.1 Baseline characteristics of the discovery cohort

The baseline demographic and clinical features of the participants are displayed in **Table 5.1**. 10 patients with ADPKD were selected and categorised into two groups according to their levels of eGFR, and 2 groups of healthy controls were selected. Initial profiling was performed on pooled urine samples in 4 groups; healthy controls (n = 10, 2 groups), ADPKD patients with eGFR > 60 ml/min/1.73 m^2 (n = 5) and ADPKD patients with eGFR < 60 ml/min/1.73 m^2(n = 5). One-way ANOVA test revealed no statistically significant differences in the mean age, gender, BMI, cholesterol, calcium, phosphate or uric acid between the 3 groups.

As expected, the eGFR at the time of recruitment was significantly lower in the ADPKD patients with eGFR < 60 ml/min/1.73 m^2 compared to other 2 groups, but was similar between healthy controls and ADPKD patients with eGFR > 60 ml/min/1.73 m^2. Similarly, mean kidney length (MKL) and eGFR slope were significantly greater in ADPKD patients with eGFR < 60 ml/min/1.73 m^2 when compared to ADPKD patients with eGFR > 60 ml/min/1.73 m^2.

Table 5.1. Clinical features of the study participants

Characteristic	Healthy controls (Mean ± SEM)	ADPKD patients with eGFR > 60 ml/min (Mean ± SEM)	ADPKD patients with eGFR < 60 ml/min (Mean ± SEM)	P value
Number	10	5	5	
Gender (F;M)	5;5	2;3	3;2	0.819
Age (years)	51.1 ± 3.758	57.2 ± 3.652	53.8 ± 4.994	0.6010
BMI (kg/m^2)	27.78 ± 1.214	27.00 ± 2.345	27.00 ± 1.208	0.9382
eGFR (ml/min per 1.73 m^2)	93.22 ± 6.98	91 ± 10.77	26.6 ± 4.885	< 0.0001
eGFR slope (ml/min per 1.73 m^2)	nm	1.150 ± 0.6278	-4.750 ± 0.5252	0.0004
MKL (cm)	nm	13.00 ± 0.7446	21.42 ± 0.9831	0.0001
Cholesterol (mmol/l)	5.480 ± 0.3203	5.060 ± 0.3957	4.840 ± 0.4946	0.4868
Uric acid (µmol/l)	356.6 ± 27.12	313.0 ± 35.90	419.5 ± 69.67	0.2882
PTH (mmol/l)	nm	36.00 ± 6.380	138.8 ± 22.15	0.0021
Calcium (mmol/l)	2.334 ± 0.01928	2.348 ± 0.04329	2.264 ± 0.01860	0.1252
Phosphate (mmol/l)	1.112 ± 0.05420	1.132 ± 0.05229	1.162 ± 0.06938	0.8439
PCR (mg/mmol)	nm	10.00 ± 1.528	23.50 ± 6.640	0.1506

The table shows the basic demographic and clinical features of the participants in the discovery profiling experiments. Estimated GFR was measured using the CKD-EPI formula. Data was analysed using one-way ANOVA test followed by a Tukey's post hoc analysis. Data was expressed as mean ± SEM. Red text highlights significant P value (< 0.05). Abbreviations are: F; Female, M; Male, BMI; Body Mass Index, MKL; Mean Kidney Length, eGFR; estimated Glomerular Filtration Rate, PTH; Parathyroid hormone, PCR; Protein Creatinine Ratio, nm; not measured.

5.5.2 Urinary angiogenesis proteomics array

The angiogenesis proteins included in the array are listed in the **Table 2.21**. Pixel intensity of each spot was quantified using Bio-Rad ChemiDoc ™XRS. The results demonstrated that the urinary profiles of angiogenesis factors between ADPKD and healthy controls were distinct (**Figure 5.1**). 2 proteins, coagulation factor III and DPPIV were detected in all the examined groups at comparable intensity. ADPKD patients with low eGFR (< 60 ml/min) excreted higher amounts of CXCL16, EGF, endostatin/collagen XVIII, MCP-1, platelet factor 4 and PLGF compared to the other groups. On the other hand, 3 angiogenesis related proteins were present at lower concentration in ADPKD with low eGFR (< 60 ml/min) compared to the other groups- serpin F1, IL-8 and endothelin-1. There appeared to be a clear trend for 4 proteins (CXC-16, MCP-1, EGF and endostatin) showing a step-wise increase from healthy controls, ADPKD patients with eGFR > 60 ml/min and those with eGFR < 60 ml/min, in contrast, 3 proteins were found to be decreased i.e. serpin F1, thrombospondin1 and IL-8. VEGF-A was increased in patients with eGFR > 60 ml/min but did not increase further in patients with eGFR < 60 ml/min. Two proteins, angiopoietin-2 and leptin, were detected only in patients with advanced ADPKD (eGFR < 60 ml/min). Taken together, the results of the discovery phase suggest that urine samples from ADPKD patients have a different angiogenesis specific signature compared to healthy controls.

Since the main purpose of this project was to identify a promising altered urine biomarker in early disease, protein expression was compared between the three groups using one-way ANOVA to analyse the data. Overall, 14 proteins were significantly altered between the examined groups, including: angiopoietin-2, CXCL16, EGF, endostatin/collagen XVIII, IL-8, leptin, MCP-1, MMP-9, PDGF-AA, platelet factor 4, PLGF, prolactin, serpin E1 and VEGF-A. The exact mean ± SEM for each protein and the P values of the group comparisons are shown in **Table 5.2**.

Because one-way ANOVA test showed significant statistical results between the groups, a Tukey's post hoc analysis was conducted to find the source of significant. The analysis showed a significant increase of angiopoietin-2, CXCL16, EGF, endostatin/collagen XVIII, IL-8, MCP-1, MMP-9, platelet factor 4, PLGF, prolactin, serpin E1 and VEGF-A in urine samples from patients with eGFR < 60 ml/min compared to other 2 groups. On the other hand, only MCP-1, endostatin, prolactin and MMP-9 showed significant increase in the patients with eGFR > 60 ml/min compared to healthy controls and patients with eGFR < 60 ml/min. Three proteins: endostatin, prolactin and MCP-1 were selected for further study.

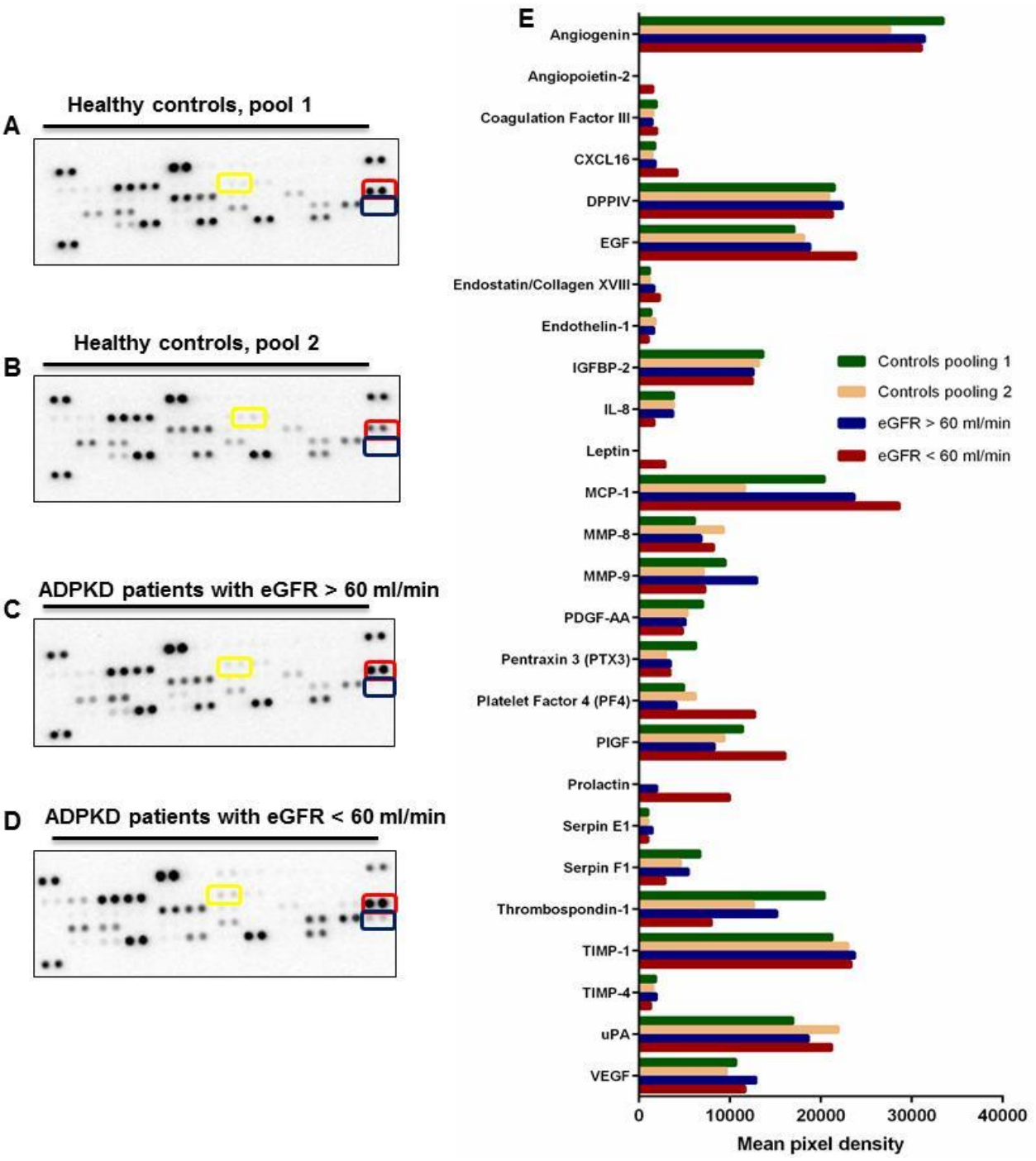

Figure 5.1. Urinary angiogenesis proteomics array

The figure presents profiling of 55 angiogenesis related proteins in; (**A**) a pooling urine sample of 5 healthy controls (pool 1), (**B**) a pooling urine sample of 5 healthy controls (pool 2), (**C**) a pooling urine sample of 5 ADPKD patients with eGFR > 60 ml/min and (**D**) a pooling urine sample of 5 ADPKD patients with eGFR < 60 ml/min. Positive and negative controls were included in each membrane, and each protein or control were represented by dark spots in duplicate. For instance, the red boxes represent MCP-1; the blue boxes correspond to prolactin, while the yellow boxes indicate endostatin expressions. **(E)** A histogram summarises the densitometries of detectable angiogenesis factor in the 4 groups.

Abbreviations are: CXCL16; C-X-C motif chemokine ligand 16, DPPIV; Dipeptidyl Peptidase IV, EGF; Epidermal Growth Factor, IGFBP-2; Insulin-like Growth Factor Binding Proteins-2, IL-8; Interleukin-8, MCP-1; Monocyte chemotactic protein-1, MMP-8; matrix metalloproteinase-8, MMP-9; Matrix Metalloproteinase-9, PDGF-AA; Platelet-derived growth factor-AA, PLGF; Placental growth factor, TIMP-1; Tissue inhibitor of metalloproteinases-1, TIMP-4; Tissue inhibitor of metalloproteinases-4, uPA; urokinase-type plasminogen Activator, VEGF-A; Vascular endothelial growth factor A.

Table 5.2. Comparison of urinary angiogenesis protein expression in ADPKD patients with eGFR > 60 ml/min, ADPKD patients with eGFR < 60 ml/min and healthy controls

Angiogenesis factors	Healthy controls (Mean ± SEM)	ADPKD patients with eGFR > 60 ml/min (Mean ± SEM)	ADPKD patients with eGFR < 60 ml/min (Mean ± SEM)	P value
Angiogenin	30404 ± 1667	31264 ± 874.3	30945 ± 1868	0.9381
Angiopoietin-2	0.0 ± 0.0	0.0 ± 0.0	308.6 ± 89.73	0.0378
Coagulation Factor III	1613 ± 85.69	1309 ± 119.4	1829 ± 124.7	0.0722
CXCL16	1499 ± 75.15	1686 ± 59.45	4070 ± 91.11	< 0.0001
DPPIV	21078 ± 410.9	22246 ± 1914	21153 ± 1577	0.7249
EGF	17471 ± 593.9	18680 ± 919.5	23745 ± 49.73	0.0031
Endostatin/Collagen XVIII	1048 ± 60.74	1523 ± 134.0	2164 ±19.14	0.0005
Endothelin-1	1970 ± 184.1	1705 ± 67.40	1494 ± 26.32	0.2415
IGFBP-2	13301 ± 286.0	12447 ± 731.0	12375 ± 331.5	0.2657
IL-8	3683 ± 30.86	3610 ±12.40	1538 ± 231.8	< 0.0001
Leptin	0.0 ± 0.0	0.0 ± 0.0	1645 ± 377.1	0.0197
MCP-1	15894 ± 2497	23528 ±166.8	28500 ± 880.1	0.0312
MMP-8	7587 ± 958.1	6697 ± 71.46	8094 ± 624.7	0.6730
MMP-9	8168 ± 663.2	12839 ± 447.9	7129 ± 9.555	0.0057
PDGF-AA	6050 ± 469.3	4957 ± 180.6	4650 ± 20.23	0.0036
Pentraxin 3 (PTX3)	4490 ± 975.7	3313 ± 5.975	3303 ± 1309	0.6501
Platelet Factor 4	5451 ± 427.0	3967 ± 27.97	12592 ± 589.8	0.0002
PLGF	10267 ± 573.1	8168 ± 473.9	15964 ± 513.7	0.0012
Prolactin	0.0 ± 0.0	1834 ± 42.94	9833 ± 557.5	< 0.0001
Serpin E1	869.9 ± 37.75	1324 ± 245.5	859.7 ± 90.92	0.0027
Serpin F1	5529 ± 783.7	5296 ± 1246	2762 ± 357.8	0.1729

Thrombospondin-1	16376 ± 2233	15026 ± 327.1	7838 ± 197.1	0.0856
TIMP-1	16830 ± 5201	23588 ± 1200	23211 ± 254.8	0.5569
TIMP-4	1566 ± 166.5	1775 ± 171.7	1159 ± 188.6	0.2112
uPA	19304 ± 1475	18498 ± 252.6	21090 ± 84.67	0.5457
VEGF-A	10006 ± 270.5	12744 ± 957.6	11542 ± 788.2	0.0386

The table shows acomparison of urinary angiogenesis proteins profiles in patients with ADPKD and the controls. Estimated GFR was measured using CKD-EPI formula. The densitometries data obtained from 4 array sets (2 arrays for the patients with ADPKD and 2 arrays for the controls), that providing 4 data measurements for each protein. One-way ANOVA followed by a Tukey's post hoc analysis was used to calculate the statistical differences among the proteins. Data presented as mean ± SEM. P values < 0.05 were considered statistically significant, and were presented with red lines in the table.

Abbreviations are: CXCL16; C-X-C motif chemokine ligand 16, DPPIV; Dipeptidyl Peptidase IV, EGF; Epidermal Growth Factor, IGFBP-2; Insulin-like Growth Factor Binding Proteins-2, IL-8; Interleukin-8, MCP-1; Monocyte chemotactic protein-1, MMP-8; matrix metalloproteinase-8, MMP-9; Matrix Metalloproteinase-9, PDGF-AA; Platelet-derived growth factor-AA, PLGF; Placental growth factor, TIMP-1; Tissue inhibitor of metalloproteinases-1, TIMP-4; Tissue inhibitor of metalloproteinases-4, uPA; urokinase-type plasminogen Activator, VEGF-A; Vascular endothelial growth factor-A.

5.6 Angiogenesis validation phase

Two proteins, endostatin and prolactin were selected for further validation in a larger cohort of 98 participants using specific ELISA. In addition, MCP-1 was included as a positive control as it has been reported to show a positive correlation with disease progression in patients with ADPKD (Zheng, Wolfe et al. 2003; Kawano, Muto et al. 2015).

5.6.1 Baseline characteristics of the validation cohort

Table 5.3 contains the clinical characteristics of the validation cohort. In total, there were 32 healthy controls (62% female), 33 ADPKD patients with eGFR > 60 ml/min (51% female) and 33 ADPKD patients with eGFR < 60 ml/min (48% female). The sample size of 66 patients was calculated based on an eGFR slope > 3 ml/min over 5 years, and it provided 80% power to detect an odds ratio of 1.9 and type 1 error (alpha level) of 0.05 using z test.

Patients with eGFR < 60 ml/min were older compared to the other groups. As expected, mean kidney length was also significantly higher in ADPKD patients with an eGFR < 60 ml/min compared to those with an eGFR > 60 ml/min.

Table 5.3. Clinical features of the study participants

Characteristics	Healthy controls (Mean ± SEM)	ADPKD patients with eGFR > 60 ml/min (Mean ± SEM)	ADPKD patients with eGFR < 60 ml/min (Mean ± SEM)	P value
Number	32	33	33	
Gender (F;M)	20; 12	17; 16	16; 17	0.490
Age (years)	42.94 ± 12.79	47.58 ± 12.68	55.52 ± 10.84	0.0003
BMI (kg/m^2)	25.26 ± 3.866	28.79 ± 5.03	27.39 ± 5.493	0.0204
eGFR (ml/min per 1.73 m^2)	99.19 ± 17.34	93.78 ± 21.76	34.88 ± 11.16	< 0.0001
eGFR slope (ml/min per 1.73 m^2)	nm	-0.3333 ± 3.833	-4.276 ± 2.894	< 0.0001
MKL (cm)	nm	13.61 ± 2.453	18.15 ± 3.335	< 0.0001
Calcium (mmol/l)	2.321 ± 0.07672	2.343 ± 0.07271	2.276 ± 0.08599	0.0038
PTH (mmol/l)	nm	41.34 ± 2.916	117.1 ±15.08	0.0001
Phosphate (mmol/l)	1.103 ± 0.1526	1.100 ± 0.1607	1.118 ± 0.1965	0.9086
Uric acid (µmol/l)	295.7 ± 83.96	308.0 ± 84.12	385.6 ± 121.4	0.0011
Cholesterol (mmol/l)	5.169 ± 0.9723	5.069 ± 1.123	4.676 ± 0.9461	0.1226
PCR (mg/mmol)	nm	10.55 ± 5.671	35.96 ± 63.33	0.0673

The table shows the basic demographic and clinical features of the participants in the angiogenesis validation experiments. Estimated GFR was measured using CKD-EPI formula. Data was analysed using ANOVA test; a Tukey's post hoc analysis was used to find the source of insignificant. Categorical variables were compared using Chi-square test the table data expressed as mean ± SEM. Dipstick urinalysis of healthy controls was negative for protein, and therefore below the detection limit and PCR levels were not measured. Abbreviations are: F; Female, M; Male, BMI; Body Mass Index, MKL; Mean Kidney Length, eGFR; estimated Glomerular Filtration Rate, PCR; Protein Creatinine Ratio, nm; not measured.

5.6.2 Analysis of urinary MCP-1, endostatin and prolactin expression in patients with ADPKD and healthy controls

Spot urine samples from healthy controls and patients with ADPKD were assayed for endostatin, prolactin and MCP-1 using commercial ELISA kits (R&D Systems UK). To adjust for diurnal variation in urine concentration, urine creatinine was used to normalize angiogenesis related proteins excretion.

The assay detection ranges of the individual ELISAs are 0-10 ng/ml for endostatin, 0-100 ng/ml for prolactin and 0-2000 pg/ml for MCP-1. Examples of each standard curve are displayed in **Figure 5.2 A**, **B** and **C** which were generated using Multiskan Ascent software (version 1.24) and the best-fit lines were calculated using a four-parameter logistic regression model. All the resulting curves were consistent with the R & D ELISA datasheet for each protein.

Figure 5.3 represents scatter plots of the urinary expression of each protein (normalized to urine creatinine) in the controls and patients with ADPKD. The expressions of MCP-1 (**Figure 5.3A**), endostatin (**Figure 5.3B**) and prolactin (**Figure 5.3C**) were comparable to their expression levels found in the discovery phase using semi-quantitative proteomics arrays. All the candidate proteins showed higher expression in the patients compared to the controls, however, only MCP-1 and prolactin showed significant P values.

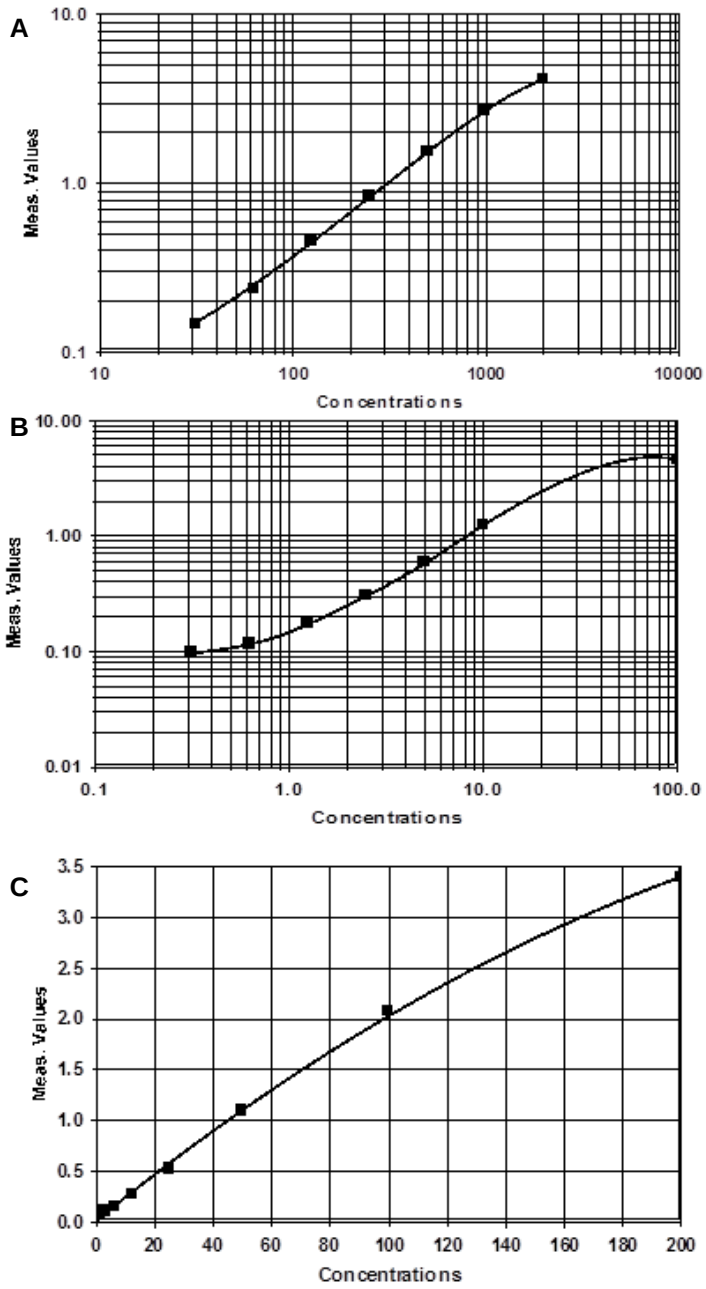

Figure 5.2. Standard curves for (A) MCP-1, (B) endostatin and (C) prolactin

The X-axis represents the protein concentration, while the Y- axis represents the absorption at 450 nm with the best-fit lines through the readings.

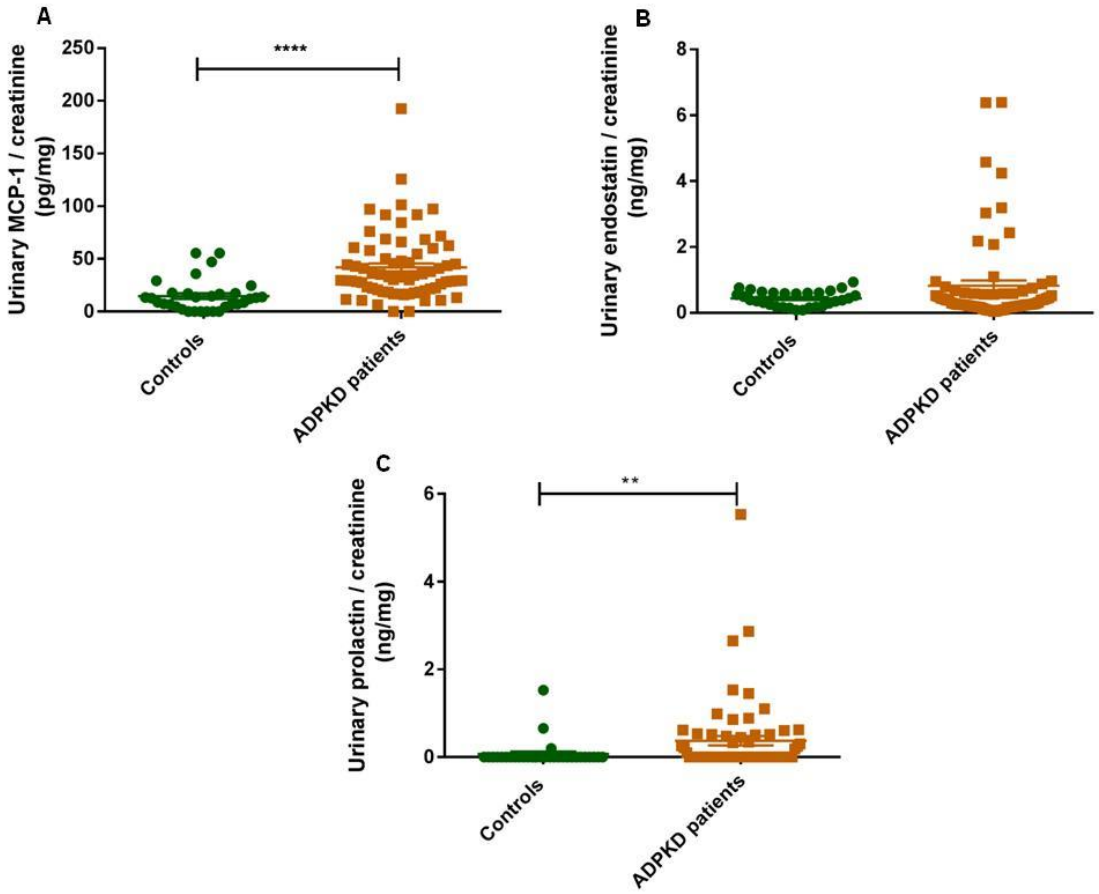

Figure 5.3. Comparison of urinary MCP-1, endostatin and prolactin expression in ADPKD patients and healthy controls

(**A**) Scatter blotting compares normalized MCP-1 expression in urine samples from patients with ADPKD (n = 66) and healthy controls (n = 32). The averages MCP-1 expression in urine was 14.63 ± 2.688 in healthy controls and 41.97 ± 3.839 in the patients group, P value < 0.0001.

(**B**) Scatter blotting compares normalized endostatin expression in urine samples from patients with ADPKD (n = 66) and healthy controls (n = 32). The averages endostatin expression in urine was 0.4339 ± 0.04212 in healthy controls and 0.8235 ± 0.1624 in the patients group, P value = 0.7909.

(**C**) Scatter blotting compares normalized prolactin expression in urine samples from patients with ADPKD (n = 66) and healthy controls (n = 32). The averages prolactin expression in urine was 0.07443 ± 0.05149 in healthy controls and 0.3743 ± 0.1058 in the patients group, P value = 0.0029.

Mann Whitney u test was used to determine the difference between the 2 groups. P value > 0.05 was considered non-significant, * denotes P value < 0.05; ** denotes P value < 0.01; *** denotes P value < 0.001 and **** denotes P value < 0.0001. Data is displayed as mean ± SEM.

To further validate the potential diagnostic role of urinary endostatin, prolactin and MCP-1 in predicting ADPKD progression, urine samples from patients with early stages ADPKD (eGFR > 60 ml/min), patients with late stage diseases ADPKD (eGFR < 60 ml/min) and healthy controls were compared using ELISA. Because one-way ANOVA test showed significant statistical results in the 3 proteins between the groups, therefore, a Tukey's post hoc analysis was conducted to find the source of significant.

The expression level of MCP-1 in urine increased gradually from normal individuals to the patients with early stages (eGFR > 60 ml/min) to the patients with advanced stages (eGFR < 60 ml/min) (**Figure 5.4A**). A 2-fold increase in MCP-1 expression was found in the urine samples from patients with early stage ADPKD as compared to the controls, but the difference was not statistically significant. A significant increase in MCP-1 expression was found in urine samples from ADPKD patients with an eGFR < 60 ml/min compared to the patients with eGFR > 60 ml/min or the controls.

The second protein studied in these validation experiments was endostatin (**Figure 5.4B**). The Tukey's post hoc analysis showed a significant increase in endostatin expression in urine samples from the patients with an eGFR < 60 ml/min compared to the patients with an eGFR > 60 ml/min and healthy controls. On the other hand, there was no significant change in endostatin expression between the patients with an eGFR > 60 ml/min compared to the controls.

Regarding prolactin, the validation ELISAs showed a consistent increase in urinary expression from healthy controls to the patients with eGFR > 60 ml/min to the patients with eGFR < 60 ml/min (**Figure 5.4C**). Although the expression of prolactin was increased more than 2-fold in urine samples from the patients with an eGFR > 60 ml/min compared to the controls, the Tukey's post hoc analysis showed that the difference was not statistically significant. Similar to that, the expression of prolactin was approximately 2-fold higher in the patients with eGFR < 60 ml/min when compared to the patients with eGFR > 60 ml/min, but it was not statistically significant. On the other hand, there was a significant difference between the controls and the patients with eGFR < 60 ml/min.

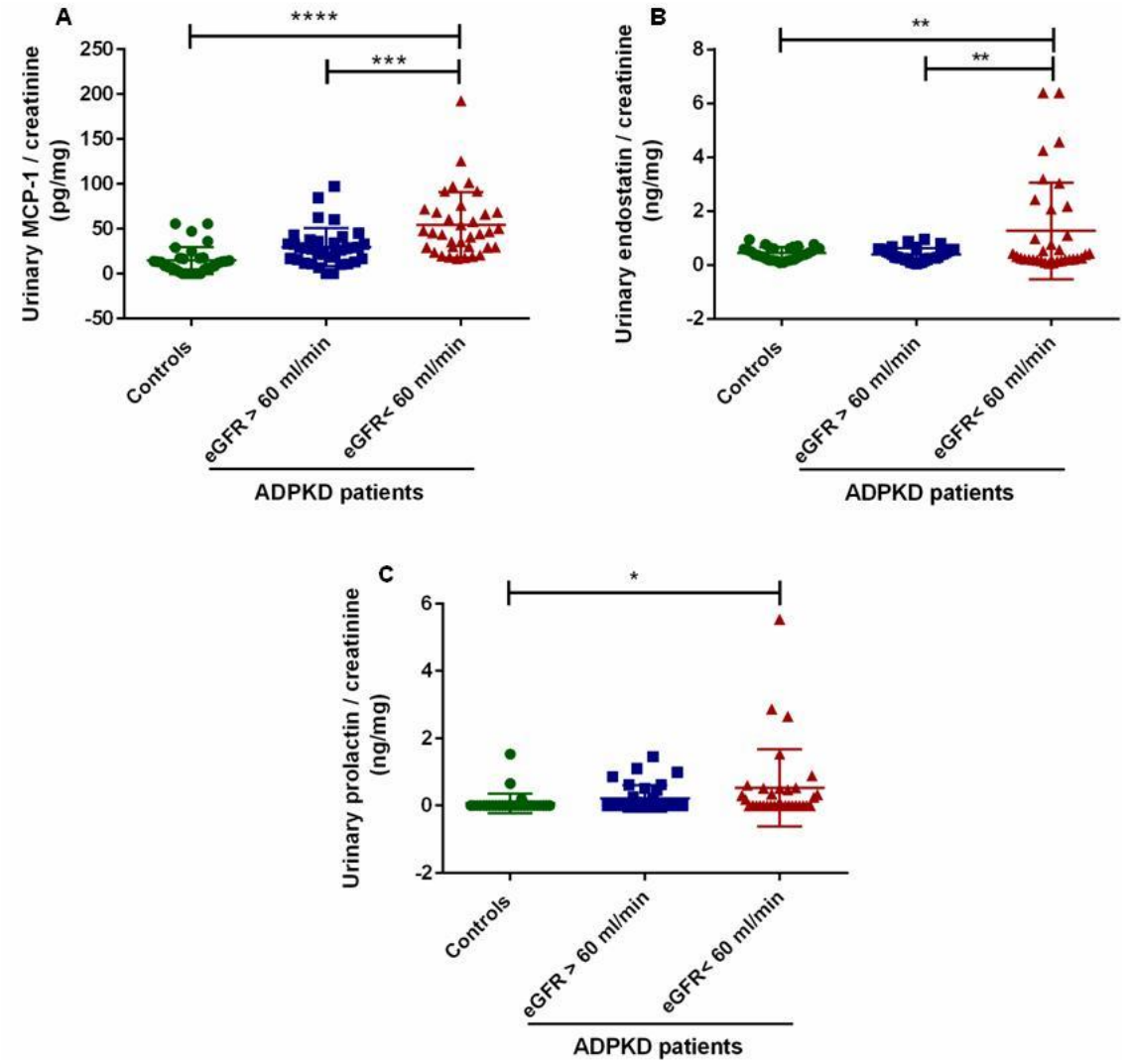

Figure 5.4. Comparison of urinary MCP-1, endostatin and prolactin expression in ADPKD patients with eGFR > 60 ml/min, ADPKD patients with eGFR < 60 ml/min and healthy controls

(**A**) Scatter blotting compares normalized MCP-1 expression in urine samples from ADPKD patients with eGFR > 60 ml/min (n = 33), ADPKD patients with eGFR < 60 ml/min (n = 33) and healthy controls (n = 32). The averages (± SEM) of MCP-1 expression in urine were 14.63 ± 2.688 pg/mg in healthy controls, 29.60 ± 3.567 pg/mg in the patients with eGFR > 60 ml/min group and 54.34 ± 6.171 pg/mg in the patients with eGFR < 60 ml/min group, P value = 0.0061. (**B**) Scatter blotting compares normalized endostatin expression in urine samples from ADPKD patients with eGFR > 60 ml/min (n = 33), ADPKD patients with eGFR < 60 ml/min (n = 33) and healthy controls (n = 32). The averages (± SEM) of endostatin expression in urine were 0.4339 ± 0.04212 ng/mg in healthy controls, 0.3876 ± 0.04088 ng/mg in the patients with eGFR > 60 ml/min group and 1.259 ± 0.3065 ng/mg in the patients with eGFR < 60 ml/min group, P value = 0.0004.

(**C**) Scatter blotting compares normalized prolactin expression in urine samples from ADPKD patients with eGFR > 60 ml/min (n = 33), ADPKD patients with eGFR < 60 ml/min (n = 33) and healthy controls (n = 32). The averages (± SEM) of prolactin expression in urine were 0.07443

± 0.05149 ng/mg in healthy controls, 0.2177 ± 0.06767 ng/mg in the patients with eGFR > 60 ml/min group and 0.5309 ± 0.1983 ng/mg in the patients with eGFR < 60 ml/min group, P value = 0.0359.

One-way ANOVA followed by a Tukey's post hoc analysis was used to determine the differences between the 3 groups. P value > 0.05 was considered non-significant, * denotes P value < 0.05; ** denotes P value < 0.01; *** denotes P value < 0.001 and **** denotes P value < 0.0001. Data is displayed as mean ± SEM.

In this project, disease progressions for the patients with ADPKD were assessed based on the rate of eGFR slope over the previous consecutive 5 years using regression coefficient analysis. This approach could overcome the fluctuation in the eGFR measurement over the observational period. Patients with an eGFR reduction of less than 3ml/min/1.73 m^2 were considered slowly progressive whereas the rapidly progressive patients were those with an eGFR decline over 3 ml/min/1.73 m^2 (Rosansky and Glassock 2014). In this section, the aim was to identify if there were any differences in the excretions of aforementioned proteins in urine samples according to eGFR slope. I compared the proteins levels in the 3 groups, patients with rapidly progressive disease (n = 28), patients with slowly progressive disease (n = 32) and healthy controls (n = 32) using one-way ANOVA. Because one-way ANOVA test showed significant statistical results in the 3 proteins between the groups, therefore, a Tukey's post hoc analysis was conducted to find the source of significance.

The greater excretions of adjusted MCP-1 were found in urine samples from the patients with rapidly progressive disease (eGFR slope > 3 ml/min), followed by the patients with eGFR slope < 3 ml/min, whereas, the lowest excretions were detected in the controls (**Figure 5.5A**). The Tukey's post hoc analysis showed a statistically significant difference in the protein excretion in the controls as compared to the patients with slowly progressive or the patients with rapidly progressive disease. However, the difference between the patients with stable disease and progressive disease was not statistically significant.

Regarding endostatin, overall, one-way ANOVA revealed significant alteration in urinary excretions among the examined groups (**Figure 5.5B**). The Tukey's post hoc analysis showed significant increase in this protein expression in urine samples from the patients with eGFR slope > 3 ml/min compared to the patients with eGFR slope < 3 ml/min and the controls. On the other hand, there was no significant change in its expressions in the patients with eGFR slope < 3 ml/min compared to the controls.

In the case of prolactin, its relative excretion in urine was increased in proportional to the disease progression (**Figure 5.5C**). The Tukey's post hoc analysis revealed significant differences in the protein expression between the patients with eGFR slope > 3 ml/min and the patients with eGFR slope < 3 ml/min as well as the controls. Although the expression of this protein in the urine samples related to the patients with eGFR slope < 3 ml/min was higher than the controls approximately 2-fold, it was not statistically difference.

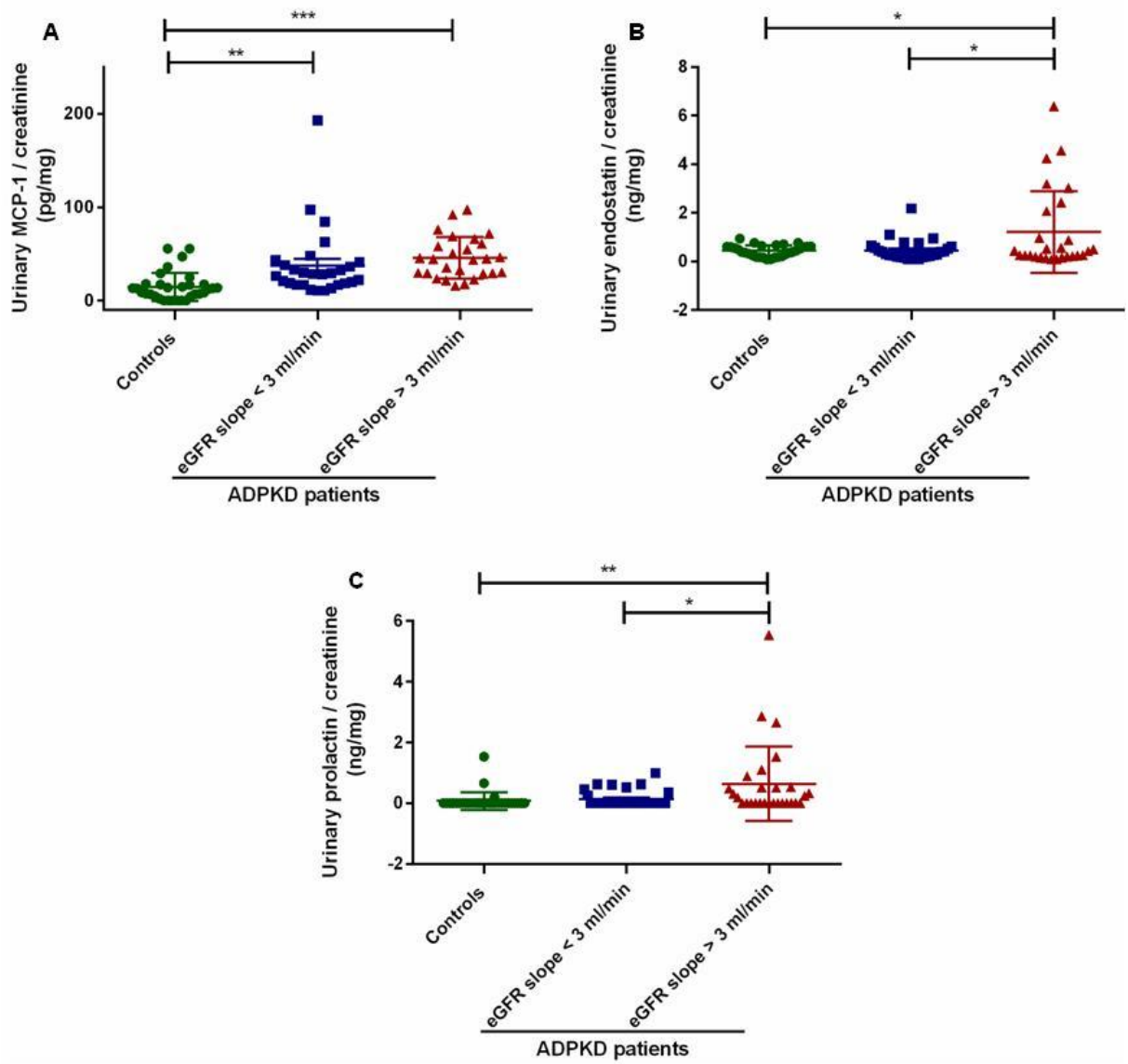

Figure 5.5. Comparison of urinary MCP-1, endostatin and prolactin expression in ADPKD patients with eGFR slope < 3 ml/min, ADPKD patients with eGFR slope > 3 ml/min and healthy controls

(**A**) Scatter blotting compares MCP-1 expression in urine samples from ADPKD patients with eGFR slope > 3 ml/min over 5 years (n = 28), ADPKD patients with eGFR slope < 3 ml/min over 5 years (n = 32) and healthy controls (n = 32). The averages (± SEM) of MCP-1 expression in urine were 14.63 ± 2.688 pg/mg in healthy controls, 37.44 ± 6.962 pg/mg in the patients with eGFR slope < 3 ml/min group and 45.87 ± 4.256 pg/mg in the patients with eGFR slope > 3 ml/min group, P value < 0.0001.

(**B**) Scatter blotting compares endostatin expression in urine samples from ADPKD patients with eGFR slope > 3 ml/min over 5 years (n = 28), ADPKD patients with eGFR slope < 3ml/min/ over 5 years (n = 32) and healthy controls (n = 32). The averages (± SEM) of endostatin expression in urine were 0.4339 ± 0.04212 ng/mg in healthy controls, 0.4483 ± 0.08181 ng/mg in the patients with eGFR slope < 3 ml/min group and 1.210 ± 0.3228 ng/mg in the patients with eGFR slope > 3 ml/min group, P value = 0.0052.

(**C**) Scatter blotting compares prolactin expression in urine samples from ADPKD with eGFR slope > 3 ml/min over 5 years (n = 28), ADPKD patients with eGFR slope < 3 ml/min over 5 years (n = 32) and healthy controls (n = 32). The averages (± SEM) of prolactin expression in urine were 0.07443 ± 0.05149 ng/mg in healthy controls, 0.1379 ± 0.04673 ng/mg in the patients with eGFR slope < 3 ml/min group and 0.6310 ± 0.2312 ng/mg in the patients with eGFR slope > 3 ml/min group, P value = 0.0064.

One-way ANOVA followed by a Tukey's post hoc analysis was used to determine the differences between the 3 groups. P value > 0.05 was considered non-significant, * denotes P value < 0.05; ** denotes P value < 0.01; *** denotes P value < 0.001 and **** denotes P value < 0.0001. Data is displayed as mean ± SEM.

5.6.3 Association of urinary MCP-1, endostatin and prolactin expression with conventional measures of ADPKD

The correlation of these urinary markers adjusted to urine creatinine with conventional measures of renal function and structure including eGFR and MKL were evaluated using Spearman correlation tests. Associations of angiogenesis factor expression levels with eGFR and MKL are depicted in **Figure 5.6** and **5.7.**

Estimated GFR is currently the baseline test for renal function assessment. All aforementioned angiogenesis factors showed negative correlations with eGFR (**Figure 5.6**). In other words, the expression of these proteins increased with disease progression. The strongest correlation was with endostatin (**Figure 5.6B**). MCP-1 also showed a significant correlation with eGFR (**Figure 5.6A**). However, urinary expressions of prolactin showed a weak correlation with eGFR (**Figure 5.6C**).

Currently, total kidney volume (measured by MRI or ultrasound) is the main clinical predictor of ADPKD progression (Grantham 2006). Mean kidney length correlates positively with total kidney volume and can be used as a measure of disease severity. Mean kidney length was positively associated with normalized urine MCP-1 levels (**Figure 5.7A**). In comparison, only weak correlations were found with endostatin and prolactin (**Figure 5.7A** and **B**).

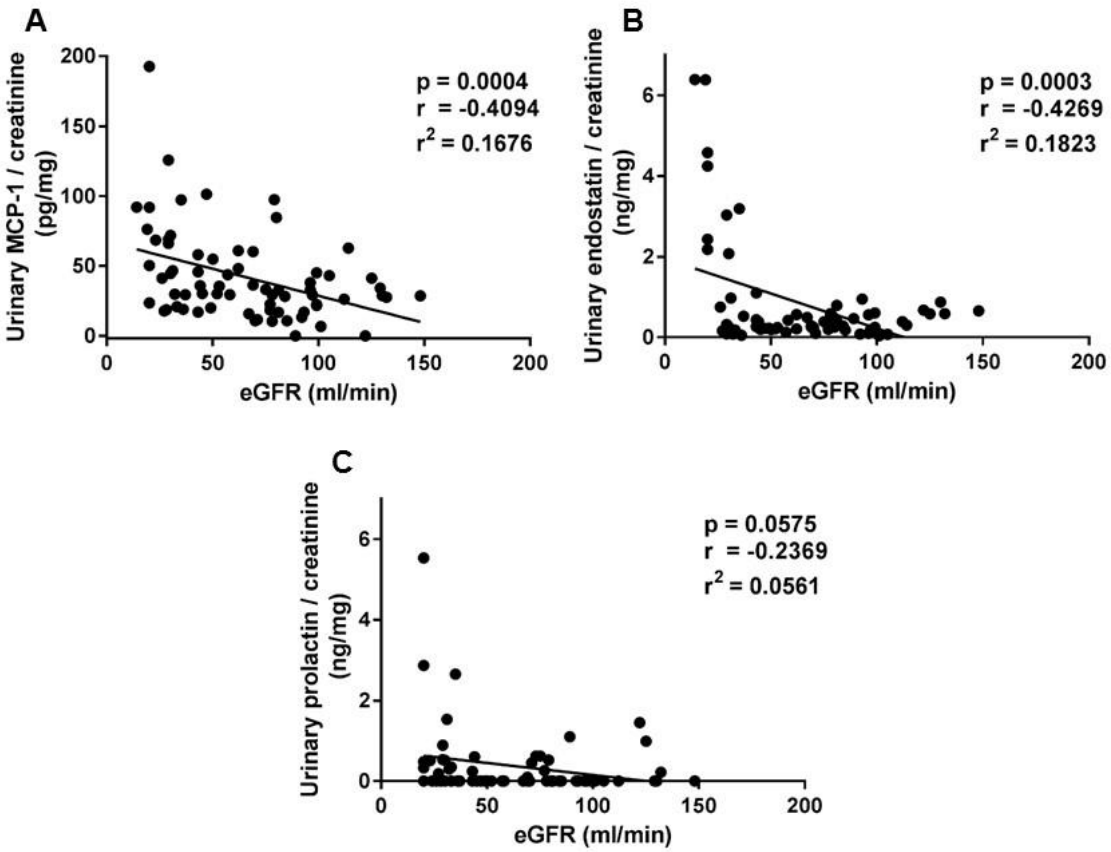

Figure 5.6. Association of urinary MCP-1, endostatin and prolactin expression with eGFR

The figure shows the correlation between (**A**) MCP-1, (**B**) endostatin and (**C**) prolactin (normalized to urine creatinine) with eGFR. For each protein, Spearman correlation coefficient (r) and coefficient of determination (r^2) were calculated with their corresponding P value. Both MCP-1 and endostatin showed significant correlations with eGFR. The solid line indicated the best correlation fits.

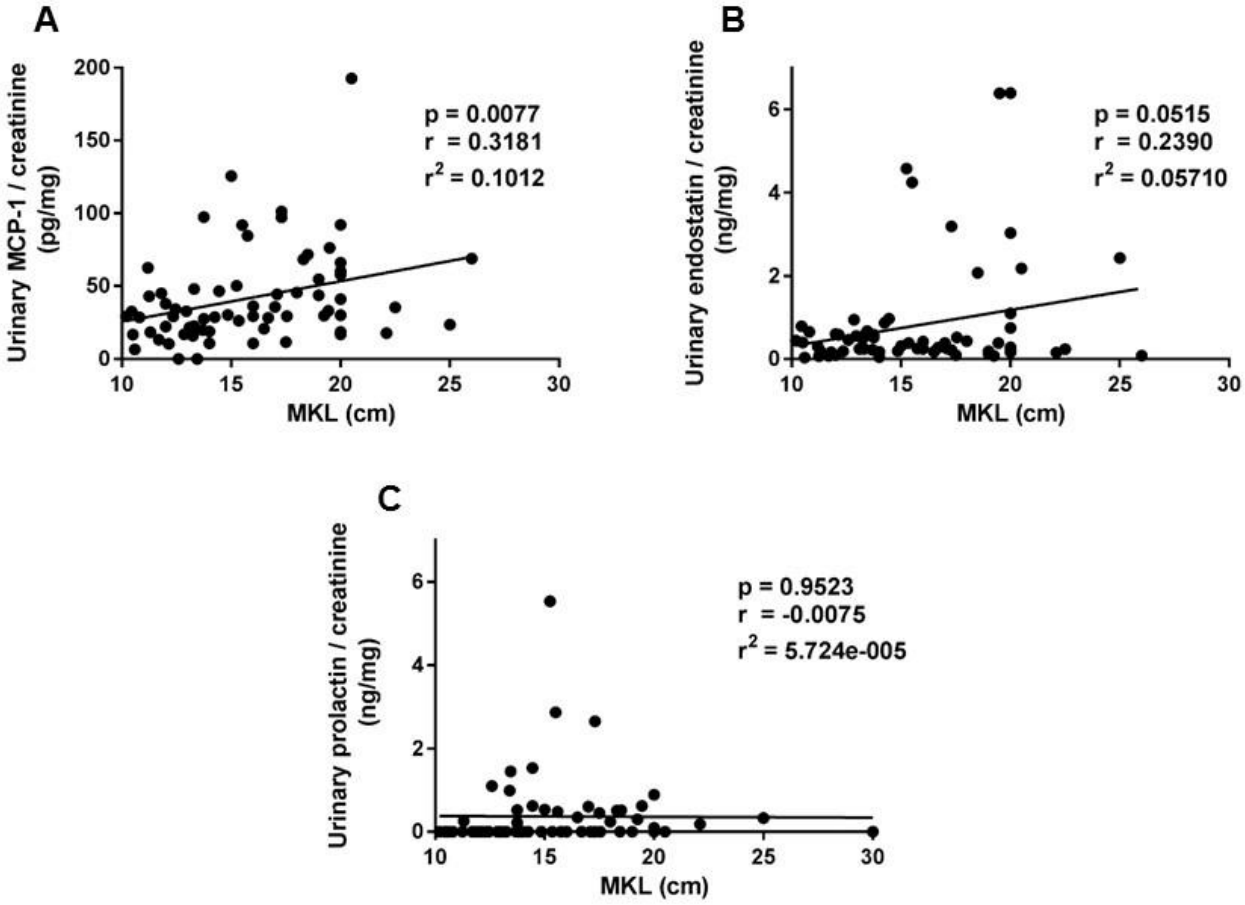

Figure 5.7. Association of urinary MCP-1, endostatin and prolactin expression with MKL

The figure shows the correlation between (**A**) MCP-1, (**B**) endostatin and (**C**) prolactin (normalized to urine creatinine) with MKL. For each protein, Spearman correlation coefficient (r) and coefficient of determination (r^2) was calculated with their P values. Only MCP-1 showed a significant correlation with MKL. The solid line indicated the best correlation fits.

5.6.4 Univariate and multivariate regression analysis

A linear regression analysis was carried to identify the correlations between the candidate proteins and other clinical variables (**Table 5.4**). No statistically significant univariate correlations were found between the examined proteins and the mean age, cholesterol and serum uric acid. Estimated GFR, BMI and PCR showed significant associations with MCP-1 and endostatin. Estimated GFR slope showed significant association with endostatin and prolactin, whereas, PCR showed significant associations with MCP-1 and endostatin. MKL and serum calcium showed significant univariate correlations with MCP-1.

All the clinical and biochemical parameters of the patients with ADPKD that revealed a significance univariate association with the examined proteins were included in a multiple regression analysis model (**Table 5.5**). Estimated GFR remained significantly associated with endostatin, whereas, only PTH showed significant association with prolactin. MCP-1 remained significantly associated with eGFR and BMI.

Table 5.4. Univariate linear regression of urinary MCP-1, endostatin and prolactin expression with clinical and biochemistry variables of ADPKD patients

Variables	MCP-1/ creatinine Standardized β (P value)	Endostatin/ creatinine Standardized β (P value)	Prolactin/ creatinine Standardized β (P value)
Age (year)	0.137 (0.297)	0.183 (0.161)	0.084 (0.505)
BMI (kg/m^2)	0.289 (0.038)	-0.271 (0.041)	-0.223 (0.086)
eGFR (ml/min/1.73m^2)	-0.483 (0.000)	-0.427 (0.000)	-0.237 (0.057)
eGFR slope (ml/min/1.73 m^2)	-0.187 (0.126)	-0.299 (0.015)	-0.324 (0.011)
MKL (cm)	0.318 (0.008)	0.239 (0.051)	-0.008 (0.952)
Cholesterol (mmol/l)	-0.153 (0.252)	-0.137 (0.305)	0.009 (0.943)
Calcium (mmol/l)	-0.277 (0.035)	-0.065 (0.629)	0.080 (0.537)
PTH (mmol/l)	0.240 (0.086)	0.535 (0.000)	0.604 (0.000)
Uric acid (µmol/l)	0.066 (0.509)	0.158 (0.254)	0.062(0.644)
PCR (mg/mmol)	0.311 (0.04)	0.447 (0.002)	0.150 (0.302)

A linear regression analysis for the various clinical and biochemical variables was generated to examine the independent associations between these parameters and normalized MCP-1, endostatin and prolactin. Standardized β (standardized coefficients beta) denotes that an alteration of 1SD in the clinical parameters (independent variable) will lead to a one measurement change in the corresponding protein (dependent factor). P value < 0.05 was considered statistically significant and denotes with red lines. Abbreviations are: BMI; Body Mass Index, eGFR; estimated Glomerular Filtration Rate, MKL; Mean Kidney Length, PTH; Parathyroid Hormone, PCR; Protein Creatinine Ratio.

Table 5.5. Multivariate linear regression of urinary MCP-1, endostatin and prolactin expression with clinical and biochemistry variables of ADPKD patients

Variables	MCP-1/ creatinine Standardized β (P value)	Endostatin/ creatinine Standardized β (P value)	Prolactin/ creatinine Standardized β (P value)
BMI (kg/m^2)	-0.347 (0.011)	-0.226 (0.145)	/
eGFR (ml/min/1.73m^2)	-0.495 (0.001)	0.497 (0.002)	/
eGFR slope (ml/min/1.73 m^2)	/	0.012 (0.947)	-0.083 (0.512)
MKL (cm)	-0.031 (0.870)	/	/
Calcium (mmol/l)	-0.219 (0.151)	/	/
PTH (mmol/l)	/	0.175 (0.371)	0.569 (0.000)
Uric acid (µmol/l)	/	/	/
PCR (mg/mmol)	0.083 (0.575)	0.218 (0.177)	/

The table shows a multivariate regression model of the selected angiogenesis proteins adjusted to the other clinical variables, which have significant correlations with the corresponding protein.

5.6.5 Prediction of ADPKD progression

Finally, the ability of these biomarkers to predict the rate of disease progression in patients with ADPKD was calculated based on eGFR slope using ROC curve analysis (SPSS). Estimated GFR slope was measured based on the decline in renal function over the past 5 years. A loss of renal function > 3 ml/min/1.73^{2}m was considered rapidly progressive disease (Rosansky and Glassock 2014). A ROC curve calculation relies on the fraction of the number of true positive (study sensitivity) and the number of false positive (1 - sensitivity).

The ROC curves of MCP-1, endostatin, prolactin and MKL are depicted in **Figure 5.8A**. Combination of ROC curves of candidate angiogenesis proteins is displaced in **Figure 5.8B**. In addition, the ROC curve of the combined proteins (MCP-1, endostatin and prolactin) with MKL were calculated (**Figure 5.8C**). The AUC, CI, and the P values of each protein, MKL, proteins combination and combination of proteins and MKL in predicting disease progression are displayed in **Table 5.6**.

The analysis revealed that MCP-1 was a significantly better predictor of disease progression than MKL. Neither endostatin nor prolactin showed a significant ability to predict disease progression in this cohort. ROC curve of protein combinations with MKL showed a significant improvement in the prediction ability of disease progression in the patients with ADPKD. Under these experimental conditions, the cut-off value of normalized MCP-1 that might be helpful in distinguishing between patients with slowly progressive disease from those with rapidly progressive disease was 28.49 pg/mg (80% sensitivity, 55.6% specificity), whereas, 14.65 ml cut-off value of mean kidney length provided 72% sensitivity and 48.1% specificity.

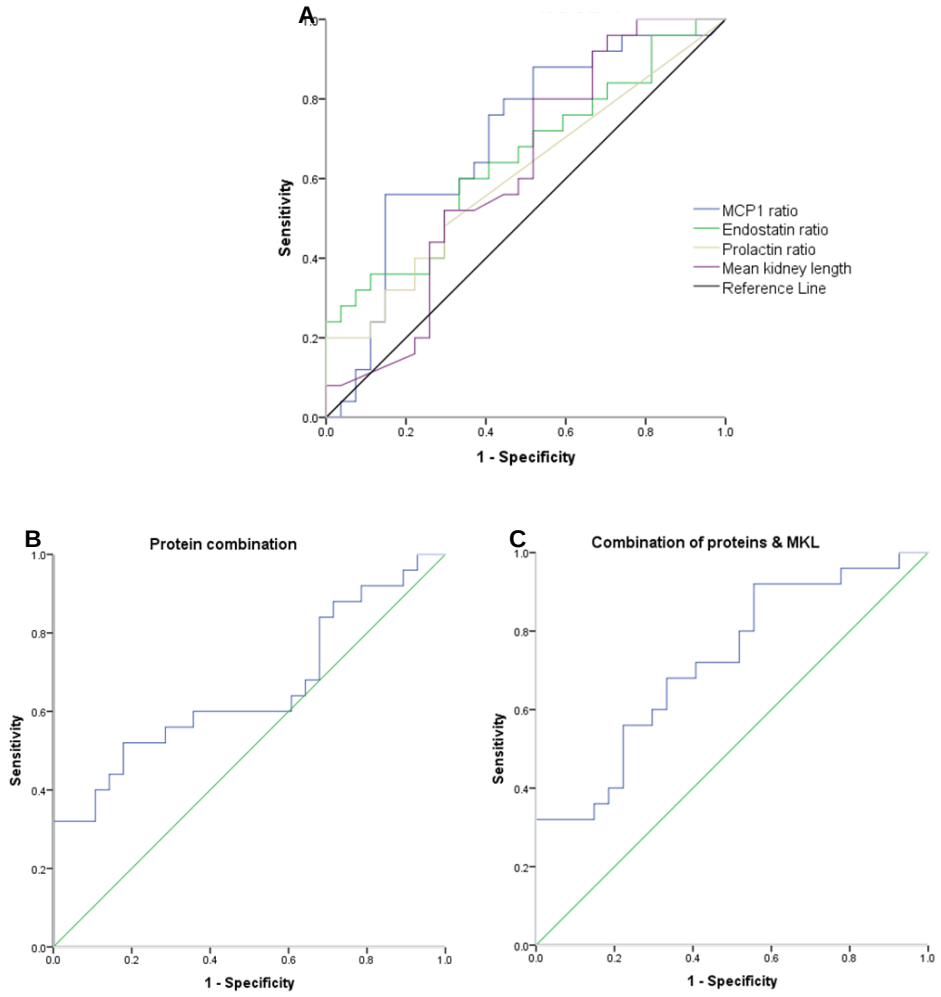

Figure 5.8. Diagnostic performance of urinary MCP-1, endostatin and prolactin to predict ADPKD progression

The figures show ROC curve for angiogenesis proteins for dichotomized eGFR slope. Combination of the angiogenesis proteins with MKL showed a higher AUC with a significant P value which reflecting a better ability to discriminate between patients with the risk of rapidly progression from the patients with slowly progressive risk compared to the MKL. Abbreviations are: ROC curve; Receiver operating Curve, MKL; Mean Kidney Length.

Table 5.6. Diagnostic performance of urinary MCP-1, endostatin and prolactin expression to predict ADPKD progression

Variables	AUC	95% CI	P value
MCP-1/ creatinine	0.695	0.554-0.846	0.013
Endostatin/ creatinine	0.646	0.498-0.799	0.066
Prolactin/ creatinine	0.604	0.450 - 0.760	0.193
Mean kidney length	0.616	0.462-0.771	0.151
Combination of proteins	0.656	0.5404-0.809	0.053
Combination of MCP-1 and MKL	0.735	0.588-0.882	0.008
Combination of proteins and MKL	0.749	0.582-0.858	0.005

Roc curve for exosomes associated proteins for dichotomized eGFR slope. MCP-1 showed the highest AUC which reflecting the ability to discriminate between patients with risk of CKD rapidly progression from patients with slowly-progressive risk. A perfect diagnostic test has AUC of 1 while a value of 0.5 indicates weak prediction ability. 95% CI indicates 95 confidences of the true AUC of the population. Red lines represent statistically significant P values, < 0.05. Abbreviations are: ROC curve; Receiver operating Curve, CI; Confidence Interval, P value; probability value.

5.7 Summary

The key findings in this chapter were:

1. A specific signature for angiogenesis related proteins was found in urine samples from patients with ADPKD compared to healthy controls.

2. Comparison of angiogenesis profiles of ADPKD patients with eGFR < 60 ml/min, ADPKD patients with eGFR > 60 ml/min and healthy controls revealed differences in the expression of angiogenesis related factors in urine.

3. Relative expression of MCP-1 and endostatin in urine were significantly correlated with function kidney test (eGFR).

4. Relative expression of MCP-1 in urine was significantly correlated with structural kidney changes (MKL).

5. High expression of MCP-1 in urine was a significantly better predictor of ADPKD progression compared to mean kidney length. In comparison, the measurement of endostatin or prolactin provided little prediction capability compared to MKL.

5.8 Discussion

In order to identify predictive urinary biomarkers of ADPKD disease progression, the complex pathophysiological processes that determine disease progression in ADPKD need to be considered. In other words, it may be necessary to consider a panel of markers instead of a single one. For instance, a large body of literature suggests that the development and expansion of kidney cysts requires vascular remodelling to support cysts growth (Wei, Popov et al. 2006). The basis for this chapter was that this could be diagnosed by measuring various urinary angiogenesis factors. In particular, I sought to identify whether changes in specific angiogenesis factors could be detected in early disease before major irreversible damage occurs.

A number of promising candidates were identified in the initial profiling phase including VEGF-A, serpin E1, thrombospondin-1, EGF, IL-8, endothelin-1, CXCL-16, MCP-1, endostatin and prolactin. From this list, 3 proteins i.e. MCP-1, endostatin and prolactin were selected for further validation based on significant changes in urine samples from patients with eGFR > 60 ml/min compared to healthy controls.

MCP-1

Our results showing that MCP-1 was significantly up regulated in urine samples from ADPKD compared to healthy controls is in agreement with a previous study (Zheng, Wolfe et al. 2003), this cross-sectional study studied MCP-1 in the serum and urine of 55 ADPKD patients and 19 healthy controls (Zheng, Wolfe et al. 2003). MCP-1 was present in cyst fluid and found to be produced by cyst epithelial cells. MCP-1 excretion was found to be increased even before increases in serum creatinine or proteinuria. These findings confirmed experimental findings in the Han:SRPD rat model, which described increased mRNA expression of MCP-1 and osteopontin in cystic kidneys compared to normal kidneys (Cowley, Ricardo et al. 2001).

A more recent cross sectional study has reported similar findings (Meijer, Boertien et al. 2010). In this study, 102 ADPKD patients and matched healthy participants were recruited. Disease severity was measured by eGFR, TKV and albuminuria. MCP-1, macrophage migration inhibitor factor (MIF), immunoglobulin G, kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) were measured in 24-hour urine samples. The results indicated that, all candidate biomarkers were significantly increased in ADPKD patients compared to the control group. In addition, TKV directly correlated with MCP-1 and KIM-1 levels (P value < 0.001). On the other

hand, I did not find any significant difference in the MCP-1 excretion in urine samples from patients with early stage ADPKD (eGFR > 60 ml/min) as compared to the controls; this result is consistent with which showed no significant difference in urine MCP-1 from ADPKD patients with normal; kidney function when compared to healthy controls (Azurmendi, Fraga et al. 2009).

MCP-1 is not only an angiogenic factor but also a potent chemoattractant for monocyte, macrophages and T cells (Deshmane, Kremlev et al. 2009). MCP-1 induces migration of memory T lymphocytes, basophils and natural killer cells, and also it mediates expression of adhesion molecules, release of enzymes and histamine by these target cells (Melgarejo, Medina et al. 2009; Kim and Tam 2011). MCP-1 mediates macrophage contribution to renal fibrosis through direct and indirect mechanisms i.e. recruitment of macrophages and also it stimulates these cells to secrete TGF-β1 (Tesch 2008; Kim and Tam 2011).

Endostatin

The second protein endostatin had not been previously studied in ADPKD although it has been shown to be a predictor of adverse clinical outcomes in other diseases. For instance, high plasma endostatin levels predict subsequent AKI in non-renal patients at intensive care unit with an AUC of 0.759 (Martensson, Jonsson et al. 2016). Plasma endostatin also predicts mortality and subsequent cardiovascular events in patients with CKD or those on haemodialysis (Carlsson, Carrero et al. 2015; Kanbay, Afsar et al. 2016). In addition, a high endostatin level has been shown to be an important poor outcome predictor in the patients with the following conditions: cardiovascular disease (Gouya, Siller-Matula et al. 2014; Ueland, Aukrust et al. 2015); pulmonary hypertension (Damico, Kolb et al. 2015); connective tissue disease (Reiseter, Molberg et al. 2015); cancer (Szarvas, Laszlo et al. 2012; Kantola, Vayrynen et al. 2014) and elderly people (Arnlov, Ruge et al. 2013).

In this project, a higher excretion of urinary endostatin was observed in the patients with eGFR < 60 ml/min compared to the patient with eGFR > 60 ml/min or the controls and could be a contributor to renal fibrosis. Higher endostatin levels in patients with CKD (eGFR < 60 ml/min/1.73^{2}m) have been reported (Chen, Hamm et al. 2012). Of interest, high endostatin levels was found to be a predictor of various causes of morbidity in patients with eGFR < 60 ml/min, however, high serum endostatin in CKD patients with preserved renal function showed lower incidence of pulmonary

complications. On the other hand, high serum endostatin in the patients with incidence of pulmonary complications provide no predictor information (Ueland, Aukrust et al. 2015; Kanbay, Afsar et al. 2016). Renal fibrosis is the main driver of ESRD in patients with CKD. Transgenic mice with endostatin over expression showed a synergistic action with TGF to increase interstitial fibrosis (Lin, Chen et al. 2016).

Prolactin

High plasma prolactin levels have been documented in a number of clinical conditions, for instance, essential hypertension (Stumpe, Kolloch et al. 1977), acute coronary syndrome (Raaz, Wallaschofski et al. 2006), platelet activation and ischemic stroke (Wallaschofski, Kobsar et al. 2004; Wallaschofski, Lohmann et al. 2006), and also predicts cardiovascular morbidity (Carrero, Kyriazis et al. 2012).

Regarding prolactin and the kidney, hyperprolactinemia is a common finding in CKD and haemodialysis patients (Ramirez, O'Neill et al. 1977; Cowden, Ratcliffe et al. 1978; Peces, Casado et al. 1979; Leu, Huang et al. 1985). A high circulating prolactin level may result from increased production or reduced renal clearance (McKenna and Woolf 1985; Caticha, Norato et al. 1996; Yavuz, Topcu et al. 2005). A high prolactin level in patients with CKD has been associated with increases in cardiovascular mortality (Carrero, Kyriazis et al. 2012). A previous clinical study showed a significant correlation between serum and urinary prolactin in lupus nephritis and with renal disease activity (Miranda, Prieto et al. 1998).

Although hyperprolactinemia may be secondary to reduced kidney clearance, it could also theoretically play a role in the pathogenesis of cystogenesis. ADPKD pathogenesis is thought to involve activation of multiple signalling pathways including cAMP/PKA, JAK/STAT, MAPK and PI3-Kinase. The main signalling pathway activated by prolactin is the JAK/STAT pathway (DaSilva, Rui et al. 1996; Radhakrishnan, Raju et al. 2012). In addition, it activates MAPK and PI3-kinase, which can further activate the mTOR pathway (Das and Vonderhaar 1996; Yamauchi, Kaburagi et al. 1998; Pauloin and Chanat 2012). Cyclic AMP pathways stimulate prolactin expression in T lymphocytes (Gerlo, Verdood et al. 2006). The role of prolactin in the pathogenesis of ADPKD required further study.

Other factors

Other candidate factors identified by the proteomics arrays as significantly altered in ADPKD urine samples but which were not further validated include VEGF-A, serpin-E1, EGF, MMP-9, PDGF-AA, IL8, CXC-16, PF4, PGF, ET-1, leptin and angiopoietin-2. Their known function and potential link with ADPKD is discussed briefly.

VEGF-A

VEGF promotes angiogenesis in physiological and pathological conditions by stimulating endothelial cells migration, proliferation and morphogenesis (Hoeben, Landuyt et al. 2004). VEGF is produced by different cell types including tumour cells (Senger, Galli et al. 1983), renal mesangial cells (Iijima, Yoshikawa et al. 1993), platelets (Verheul, Hoekman et al. 1997) and macrophages (Sunderkotter, Steinbrink et al. 1994). Circulating levels of VEGF, beside other angiogenesis growth factors, have been studied in ADPKD patients with preserved renal function (mean age 16 years and eGFR > 60 ml/min/1.73 ^{2}m) (Reed, Masoumi et al. 2011). They report an increase in serum VEGF in ADPKD compared to age matched patients with diabetes and preserved renal function. Consistent with this study, we found a higher expression of urinary VEGF in patients with preserved renal function compared to controls. In our results, however, the level of urinary VEGF was lower in patients with eGFR < 60 ml/min compared to the patients with eGFR > 60 ml/min. Serum levels of VEGF were higher and increased in the kidney of a Han:SPRD rat (4 weeks old) compared to wild type (Tao, Kim et al. 2007) whereas renal VEGF expression was lower in a Han:SPRD rat (12 weeks age) compared to its expression in non-cystic animals (Stringer, Komers et al. 2005). This result has been interpreted as active vascular growth in the earliest stages of cyst expansion requiring higher VEGF expression; however, with advanced stages of the disease, blood vessels are lost and this results in a lower level of VEGF in the kidneys (Huang, Woolf et al. 2013). A similar result was found in microarray-based gene-expression profiling of VEGF in human proximal tubular epithelial cells (Rudnicki, Perco et al. 2009). They showed that VEGF transcription was down regulated in cells from CKD patients with progressive proteinuria in comparison to patients with a stable disease. Recently, a single nucleotide polymorphism (SNP) study of the VEGF gene in 302 participants (73 patients with ADPKD and 229 controls) examined the influence of VEGF polymorphism on ADPKD progression (Martins, Souza et al. 2016). The authors did not find any difference in the frequency of the VEGF-C936 T allele between controls and patients. However, in terms of disease progression, the presence

of the T allele in the gene appeared to accelerate ADPKD progression and moreover, it seemed to be an anticipatory biomarker of disease progression (Martins, Souza et al. 2016).

Blockade of VEGF expression using a specific antibody in the Han:SPRD rat model of ADPKD led to a significant reduction in tubular proliferation and cyst growth, which resulted in a reduction in cystogenesis and further prevention of renal function decline (Tao, Kim et al. 2007). In addition, anti-VEGF treatment retards growth of *PKD2* mice liver cysts (Amura, Brodsky et al. 2007). However, another study examining the effects of anti-VEGF in Han:SPRD rats reported opposite results (Raina, Honer et al. 2011). The author's administrated anti-VEGF–A antibody intraperitoneally to 4 weeks old wild-type and cystic Han:SPRD male rats for 6 weeks. This resulted in kidney injury with worsening of proximal tubular cysts.

Serpin E1 (Plasminogen activator inhibitor-1)

Another protein, serpin E1, belongs to the serine protease inhibitor gene family (Eddy 2002). Regulation of vascular fibrinolysis is the main function of serpin E1, besides that, it has a role in extracellular remodeling, pro-fibrotic effects and tumor proliferation (Rerolle, Vigneau et al. 2001). Under physiological conditions, serine E1 is present at low serum concentrations with a short half-life. Higher serum levels are reported to be a risk factor for tumor growth and metastasis, thromboembolic diseases, and atherosclerosis (Durand, Bodker et al. 2004; Arenillas, Alvarez-Sabin et al. 2008; Tjarnlund-Wolf, Brogren et al. 2012).

In this project, urinary serpin E1 showed a significant elevation in the patients with eGFR > 60 ml/min as compared to the patients with eGFR < 60 ml/min or the controls, P value = 0.0027. Higher expression of serpin E1 in cystic epithelial cells has been reported in human and mouse lines (Hassane, Leonhard et al. 2010; Norman 2011). However, contrary to our result, one study found higher expression of serpin E1 in a *PKD1* mouse with advanced disease (4 months) compared to animals with earlier disease (14 days, 1, 2 and 3 months). Renal serpin E1 expression has also been reported to be higher in patients with other forms of CKD. Here, it may play a role in the pathogenesis of glomerulonephritis, diabetic nephropathy and chronic allograft rejection (Tomooka, Border et al. 1992; Tang, Friess et al. 1998; Nicholas, Aguiniga et al. 2005).

Epidermal growth factor (EGF)

Interestingly, urinary EGF was higher in patients with eGFR < 60 compared to other participants. The first link between EGF and PKD pathogenesis came from experimental models. In 1998, it was demonstrated that changes in EGF receptor activity contributed to kidney cyst formation in the orpk mouse (Richards, Sweeney et al. 1998). In a recent study, several EGF receptor ligands including TGF-α were studied in urine and blood from 27 patients with ADPKD on tolvaptan treatment and 27 controls using ELISA (Harskamp, Gansevoort et al. 2015). They measured the ligands at baseline, 3-weeks after treatment with Tolvaptan, and 3 weeks after treatment withdrawal. They concluded that higher levels of urinary EGF ligand excretion were associated with severe disease. The role of EGF in the regulation of proliferation of ADPKD and normal epithelial cells has been previously reported (Zheleznova, Wilson et al. 2011). Of interest, EGF expressions in urine were reported to be lower in the patients with various CKD aetiology including DM, AKI, IgA nephropathy and glomerulonephritis (Klein, Bascands et al. 2016).

Matrix metalloproteinase-9 (MMP-9)

MMP-9 belongs to the matrix metalloproteinase (MMP) family and has a pro-fibrotic role in epithelial–mesenchymal transition (Tan, Zheng et al. 2013). In our study, urinary MMP-9 was significantly higher in patients with eGFR > 60 ml/min compared to the other groups (P value = 0.0057). In Zeisberg et al. study, MMP-9 expression was detected mainly in the renal tubules of a normal mice, but MMP-9 expression spread to the glomeruli of a mice model of the disease by 4 weeks, which further spread to the interstitial compartment by 8 weeks of age (Zeisberg, Khurana et al. 2006). That study results suggested that enhanced MMP9 family activity was detectable in as stage specific pattern of MMP-9, beside other members of the family, prior to the onset of proteinuria and it could has a role in the progression of renal diseases in a mice model of Alport syndrome enhanced MMP9 family activity was detectable prior to the onset of proteinuria (Zeisberg, Khurana et al. 2006), whereas, in the later stages, decreased MMP expression levels, together with a higher expression of their inhibitors TIMP-1 and 2, were found in human and animal models of kidney fibrosis (Eddy 1996; Zeisberg, Khurana et al. 2006). High expression of MMP-9 in cystic tissue has been reported (Liu, Li et al. 2012).

Platelet derived growth factor-AA (PDGF-AA)

Our results also reveal a clear trend of reducing PDGF-AA expression from healthy controls to patients with early stages CKD, to the patients with advanced CKD, P value = 0.0036. PDGF-AA is a member of platelet-derived growth factor family (Buhl, Djudjaj et al. 2016). ADPKD has been classified as a ciliopathy (Yoder 2007; Patel, Chowdhury et al. 2009), and several cilia localized receptors have been recognized including PDGF-AA receptor (PDGF-αα) (Gerdes, Davis et al. 2009; Habbig and Liebau 2015). Alteration in ciliary function could lead to a deregulation of PDGF-αα signalling (Schneider, Clement et al. 2005; Nigg and Raff 2009).

Interleukin-8 (IL-8)

Prior studies reported the presence of inflammatory markers in the cystic fluid of human and animal models of ADPKD (Karihaloo, Koraishy et al. 2011). For instance, TNF alpha, IL-1 beta and IL-2 were found in renal cysts, whereas CXCR-2 agonists were found to be highly expressed in the fluid of a liver cyst (Gardner, Burnside et al. 1991; Amura, Brodsky et al. 2008). In our study, the chemokines MCP-1, IL-8 and CXCL-16 were differentially expressed in the discovery array.

IL-8 is a pro-inflammatory cytokine. A previous study reported that IL-8 was present in high concentrations in human liver cystic fluid and is secreted by cystic epithelial cells (Nichols, Gidey et al. 2004). Moreover, this cytokine can promote cystic cell proliferation (Amura, Brodsky et al. 2008). The serum concentration of IL-8 and other cytokines factors are higher in patients with ADPKD compared to controls (Merta, Tesar et al. 1997). In addition, secretion and expression of IL-8 in cystic lines of human ADPKD are higher than the normal cells; therefore inhibition of this chemokine receptor could inhibit cellular proliferation (Lee, Song et al. 2014).

In our study however, urinary IL-8 levels were lower in ADPKD patients with late stage CKD. Our findings are in agreement with a study in patients with diabetic nephropathy (DN) (Tashiro, Koyanagi et al. 2002). Tashiro et al. showed that urinary MCP-1 increased with stages of DN, while IL-8 showed lower expression of the advanced disease compared to the early stages. The authors suggested that the differences in MCP-1 and IL-8 is due to their different role in the pathogenesis of DN. Urinary IL-8 levels have been reported to be increased in the acute stages of different

glomerular diseases and in IgA-nephropathy (Wada, Yokoyama et al. 1994; Yokoyama, Wada et al. 1998).

CXC-16

CXC-16 was another chemokine which showed a step-wise upregulation from healthy controls to early ADPKD showing the highest expression in ADPKD patients with later disease. *PKD1* null cells have been reported to produce higher concentrations of CXC-16 and MCP-1 which in turn can stimulate macrophage and inflammatory cell infiltration (Karihaloo, Koraishy et al. 2011). High serum CXC-16 correlated with the activity of lupus nephritis in human and animal models and has been proposed as a predictive biomarker (Wu, Xie et al. 2007; Qin, Guo et al. 2014).

Platelet factor-4 (PF4)

In this project, higher expression of platelet factor-4 (PF4) was detected in urine samples from patients with eGFR < 60 ml/min compared to the patients with eGFR > 60 ml/min or healthy controls. PF4 is a CXC chemokine and it participates in various biological processes including angiogenesis, atherosclerosis and inflammation (Brandt, Ludwig et al. 2000; Bikfalvi 2004; Lambert, Rauova et al. 2007). However, the role of this protein in the pathogenesis of ADPKD has not yet been investigated. Of interest, expression of PF4 has been shown to be upregulated in the early stages of liposarcoma and adenosarcoma by mass spectrometry (Cervi, Yip et al. 2008).

Placental growth factor (PLGF)

Placental growth factor (PLGF) was highly expressed in patients with advanced CKD compared to the other 2 groups. This finding is in accordance with Zakiyanov et al. results which showed higher expression of PLGF in 45 CKD patients (Peiskerova, Kalousova et al. 2013), 6 of them have ADPKD, compared to controls using ELISA (Zakiyanov, Kalousova et al. 2011). Interestingly, PLGF was reported to be a potential diagnostic marker for increased left ventricular mass index in patients with CKD; left ventricular index is a strong predictor of ESRD in patients with non-diabetic CKD (Paoletti, Bellino et al. 2011; Peiskerova, Kalousova et al. 2013).

Endothelin-1 (ET-1)

Several papers have linked renal endothelin-1 (ET-1) to interstitial fibrosis, apoptosis and cyst growth in ADPKD. Over-expression of ET-1 in human ADPKD cystic kidney or in a mouse model of ADPKD has been reported (Nakamura, Ebihara et al. 1993; Chang and Ong 2011). In addition, ET-1 transgenic mice develop renal fibrosis, glomerulosclerosis, apoptosis and cyst development in an age dependent manner (Hocher, Thone-Reineke et al. 1997; Hocher, Rohmeiss et al. 1998). Our study however showed lower urine ET-1 levels in patients with ADPKD compared to the controls. In a recent study of 20 patients with ADPKD, a negative correlation of urinary ET-1 excretion and eGFR but a positive correlation with ACR and MKL was reported (Raina, Lou et al. 2016).

Leptin and angopoietin-2

Two proteins were only expressed in the urine from patients with advanced disease (Leptin and angiopoietin-2). Therefore, these markers could be a reflection of late stages renal disease rather than the process of cystogenesis. In the case of angiopoietin-2 (a member of vascular growth factor family), this idea was supported by Tsai et al. and Dane et al. studies in CKD (Woolf, Gnudi et al. 2009; Dane, Khairoun et al. 2014; Tsai, Chiu et al. 2014). Moreover, angio-2 has been suggested as an independent predictor factor of mortality in patients with ADPKD, CKD, on dialysis or kidney transplant (David, Kumpers et al. 2009; Reed, Masoumi et al. 2011; David, John et al. 2012; Molnar, Kumpers et al. 2014; Tsai, Lee et al. 2015).

Regarding leptin, it is a multi-functional peptide hormone, which has a role in weight regulation, angiogenesis, and immune system modulation (Considine, Sinha et al. 1996; Wolf, Chen et al. 2002). Circulating levels of leptin tend to increase in patients with advanced CKD (Widjaja, Kielstein et al. 2000; Briley and Szczech 2006). This can be explained by reduction of leptin clearance by the kidneys due to a reduction in glomerular filtration rate (Mak, Cheung et al. 2006; Zhang and Wang 2014).

Chapter 6 General discussion

Although the median age at ESRD in ADPKD is 55 years of age, there can be significant intrafamilial and interfamilial variability (Harris and Rossetti 2010). The causes of this variability relate to a number of biological, genetic and environmental factors (Helal, Reed et al. 2012; Ong, Devuyst et al. 2015). In addition, renal function may remain almost normal in patients with ADPKD during the early and middle phases of disease due to compensatory hyperfiltration by non-cystic nephrons. The measurable decline in renal function occurs late, therefore, the challenge of early identification and treatment of ADPKD patients at highest risk of developing ESRD is an important clinical goal. Although genotype provides one way of stratifying patients, the current cost of genotyping remains prohibitive for routine clinical use.

In recent years, much effort has gone into identifying accurate and sensitive non-invasive prognostic biomarkers of disease progression in patients with ADPKD (Schrier, Brosnahan et al. 2014; Corradi, Gastaldon et al. 2016). However, the nature of disease and its long duration have made it difficult so far to identify a single prognostic biomarker. Total kidney volume (TKV) determined from MR measurements is currently the most widely accepted imaging biomarker but is not yet routine in clinical practice. For instance, height-adjusted TKV (600 cc/m) predicted the onset of stage 3 CKD (measured GFR less than 30 ml/min/1.73m^2) within 8 years (74% sensitivity and 75% specificity) in the observational CRISP cohort of patients enrolled with an entry GFR of > 70 ml/min (Chapman, Bost et al. 2012). A number of serum and urine biomarkers have also been reported in the literature (**Table 1.3**); however, most of these reported are lack of sensitivity and specificity measurements. NGAL, urine fetuine-A, KIM-1 and IL-18 are nonspecific for ADPKD.

In this project, a number of proteins, microRNAs and angiogenic factors were measured in urine samples from patients and healthy controls to discover novel biomarkers for disease progression in ADPKD. Due to the low abundance of microRNAs detectable in whole urine during pilot experiments, methods were developed to isolate urinary exosomes for this purpose.

Urinary exosome associated proteins

In the first part of the project, I examined the expression of PC1 and PC2 in urinary exosomes. The quantity of exosomes excreted in urine has been reported to be similar in ADPKD patients with different stages of disease and healthy controls (Yuana, Sturk et al. 2013; Pocsfalvi, Raj et al. 2015). The levels of exosome associated PC1 and PC2

(assessed by Western blotting) were significantly lower in patients regardless of their renal function when compared to healthy controls (**Chapter 3**). Although the genotype of the patients studied was not determined, it is likely that *PKD1* mutations account for 80-85% in this cohort and *PKD2* mutations for the rest (Pei and Watnick 2010). Thus it was surprising that a reduction in the expression of both proteins was measured. There is however good experimental evidence that PC1 and PC2 usually function as partners in a heterodimeric protein complex and also that one may stabilise the expression of the other (Fedeles, Tian et al. 2011). My result confirmed Hogan et al. study that reported a reduction (but not absence) in PC1 and PC2 expressions in ADPKD urine exosomes compared to healthy controls by MS based proteomics analysis (Hogan, Bakeberg et al. 2015), but does not supported Pocsfalvi et al. report that PC1 and PC2 expressions are absent in urine samples of ADPKD patients compared to healthy controls using ITRAQ labelling mass spectrometry and western blotting (Pocsfalvi, Raj et al. 2015).

Of interest, univariate and multivariate analysis showed that exosomal PC1 (but not PC2) expression was an independent predictive factor for disease progression. However, both proteins did not outperform conventional measures of mean kidney length as measured by ROC analysis. I was also unable to confirm the presence of smaller mutant bands of either protein in exosomes which might have been predicted from cellular studies (Ong, Harris et al. 1999; Qian, Boletta et al. 2002).

Because of other work from our group showing the upregulation of the EGF receptor family member, ErbB4, in cystic cells and tissues, I next examined the expression of ErbB4 in exosomes and its potential role in predicting disease progression. ErbB4 is a member of ErbB/HER family of protein-tyrosine kinases that plays a regulatory role in cellular proliferation, differentiation, migration, and also renal tubular formation (Yarden and Sliwkowski 2001; Zeng, Zhang et al. 2007; Veikkolainen, Naillat et al. 2012). In comparison to PC1 and PC2, active ErbB4 expression (cleaved C-terminal fragment) was significantly elevated only in patients with late disease (GFR < 60 ml/min) in relation to healthy controls and patients with early disease (GFR > 60 ml/min). In ROC analysis, ErbB4 had significant predictive value for more rapid disease progression (AUC 0.745). Significantly, the combination with ultrasound measured mean kidney lengths (AUC 0.698) further improved predictive ability (AUC 0.816) suggesting that both markers are independent prognostic factors measuring different aspects of disease progression. This is the first report of urine exosomal ErbB4 expression in the literature and its correlation with the rate of disease progression in any renal disease.

Urinary exosome associated microRNAs

Urinary microRNAs represent potentially useful disease biomarkers and have been studied in a range of kidney diseases including ADPKD (**Table 1.6** and **1.7**). In pilot experiments (**Chapter 4**), I found that the detection of microRNAs in whole urine was too low to provide consistent results. Further work therefore focussed on microRNA expression in exosomes. Since little was known about differential expression of microRNAs in urinary exosomes, next generation sequencing was first conducted in a discovery cohort of patient and control samples. After bioinformatics analysis, 6 differentially deregulated microRNAs i.e. miR-30a-5p, miR-30d-5p, miR-30e-5p, miR-192-5p, miR-193b-3p and miR-194-5p, were selected for further validation in a larger cohort of 20 patients with early disease (eGFR > 60 ml/min), 20 patients with late disease (eGFR < 60 ml/min and 20 healthy controls by TaqMan qPCR assays. These results confirmed that all 6 microRNAs were down-regulated in ADPKD exosomes relative to healthy controls. Of relevance, all the validated microRNAs, except miR-194-5p, could discriminate between slow and rapid disease progression with individual AUC ranging from 0.7 to 0.8 in ROC analysis. The combination of all microRNAs and MKL further improved AUC to 0.94 outperforming ErbB4 suggesting that they are independent factors.

Of interest, multivariate regression analysis also revealed significant correlations between mir-192-5p and MKL, and also between mir-193b-3p and eGFR slope. Mir-192-5p was the only microRNA to show a significant change in early disease relative to controls whereas mir-193b-3p was altered in late disease but had the highest AUC value in predicting rapid disease progression. These findings suggest that they could reflect disease processes important in early and late disease respectively.

The specific pathways that could be regulated by these microRNAs were not determined in this project with the exception of a link between miRNA-193b-3p and ErbB4. However, pathway enrichment analysis suggested that potentially 11 signalling pathways could be implicated. In turn, these pathways could play important roles at different stages of disease affecting cellular processes such as proliferation, differentiation, migration and apoptosis. For instance, the miR 30 family is known to be involved in epithelial-mesenchymal transition, calcium/calcineurin signalling, P53 signal, Wnt signalling pathways, TGF-β signalling, apoptosis and proliferation (Braun, Hoang-Vu et al. 2010; Li, Donath et al. 2010; Shi, Yu et al. 2013; Zhao, Lin et al. 2014; Wu, Zheng et al. 2015). MiR-192 has been shown to be involved in TGF-beta/Smad3-signal and interstitial fibrosis, as well as in tubular sodium transport (Elvira-Matelot, Zhou et al. 2010; Krupa, Jenkins et al. 2010). In addition, miR 192-5p and 194-5p

regulate p53 expression, a transcription factor which has been implicated in the pathogenesis of ADPKD (Nishio, Hatano et al. 2005; Van Bodegom, Saifudeen et al. 2006; Pichiorri, Suh et al. 2016). Mir-194 supresses cellular proliferation, migration and invasion of cancer cells and low expression of this microRNAs has been associated with tumour metastasis (Zhao, Ren et al. 2014; Chen, Wang et al. 2015; Zhang, Zhuang et al. 2016). Finally, miR-193b-3p has been linked to the regulation of cyclin D1, MAPK signal activity, TGF-β signalling and receptor tyrosine kinases signalling (Chen, Feilotter et al. 2010; Ikeda, Tanji et al. 2012; Haetscher, Feuermann et al. 2015; Hou, Yang et al. 2015; Kaukoniemi, Rauhala et al. 2015; Zhou, Li et al. 2016).

This study has not shown the potential for urine exosome mRNA as disease biomarkers in ADPKD. However, it is possible that other altered mRNAs could be present in cellular compartments other than exosomes and therefore not detected. Finally, it is possible that the primary effect of the microRNAs was mediated through inhibiting mRNA translation (and hence protein levels) rather than through altering mRNA stability (and mRNA levels). Further studies will be required to clarify these points.

Urinary angiogenic factors

Previous studies have shown that major changes in the kidney vasculature occur in the ADPKD kidney even at the earliest stages of disease (Bello-Reuss, Holubec et al. 2001; Wei, Popov et al. 2006; Huang, Woolf et al. 2013). In the final chapter, I sought to identify changes in urine factors that might be useful to differentiate ADPKD patients from healthy control subjects (**Chapter 5**). In the discovery phase using an antibody array of 55 angiogenic factors and 4 groups of pooled urine samples revealed 14 deregulated factors in patients compared to controls. Three factors (MCP-1, endostatin and prolactin) were then chosen for individual validation by ELISA in a cohort of 98 participants.

Overall, there were higher urinary concentrations of factors in patients compared to healthy controls. In the validation cohort, the excretion of MCP-1 and prolactin (but not endostatin) was confirmed as significantly increased in ADPKD patients. MCP-1 levels were negatively correlated with eGFR and positively correlated with MKL. ROC analysis showed that MCP-1 was able to predict rapid disease progression (GFR slope > 3 ml/min), outperforming MKL. In contrast, prolactin and endostatin were not found to be sensitive predictors of rapid disease progression.

These results confirm the potential of urine MCP-1 as a potential biomarker of disease progression in ADPKD as has been shown by other groups (Zheng, Wolfe et al. 2003; Meijer, Boertien et al. 2010). MCP-1 could have a specific role in promoting tubule-interstitial inflammation through macrophage recruitment (Viedt, Dechend et al. 2002). The source of MCP-1 in the ADPKD kidney was not determined in this study but could originate from cystic epithelial cells (Cowley, Ricardo et al. 2001; Zheng, Wolfe et al. 2003). Clearly, MCP-1 itself is not specific to ADPKD and could be increased by other confounding inflammatory or infective processes (Hanemann, Liborio et al. 2013).

Concluding remarks

Although a large number of molecules have been reported as potential diagnostic or prognostic biomarkers, very few have so far been validated for clinical use (Poste 2011; Drucker and Krapfenbauer 2013). For instance, serum PSA has been successfully used for monitoring of prostatic cancer progression (Lilja, Ulmert et al. 2008). Another example is urinary nuclear matrix protein-22, approved by the FDA as a qualitative diagnostic test for monitoring bladder cancer (Drucker and Krapfenbauer 2013; Goodison, Rosser et al. 2013).

There are several important issues that need to be addressed before a discovery biomarker can be translated into clinical practice (Drucker and Krapfenbauer 2013). A discovery biomarker should be reproduced in a large validation cohort comprising a range of age, ethnicity and disease stages with high sensitivity and specificity (Drucker and Krapfenbauer 2013). Availability of a suitable clinical assay and development of a comprehensive protocol for sample collection, storage and data analysis are key issues in the validation stage (Drucker and Krapfenbauer 2013).

In this study, I have identified several proteins, microRNA and cytokines as potential disease biomarkers in ADPKD. Strengths of the study include the prospective nature of the study with fresh sample collection, a well characterised cohort with good matching of patients and controls, the ability to correlate changes with historical or recent clinical measures of disease progression, and a discovery approach especially with microRNA and angiogenic factors followed by a validation phase. I was able to validate findings from cell culture studies linking ErbB4 and mir-193b-3p although the link between the other deregulated microRNAs and predicted mRNA targets remains unproven. Study limitations were that (1) this was a single centre study with participants sampled at a single time point; serial measurements were not performed. (2) Serum samples were

not analysed so the possibility of overspill from the circulation cannot be excluded, except for exosomes. (3) Urine expression was not compared between ADPKD and other forms of CKD in this study; therefore, the changes detected may not be specific to ADPKD but reflect the stage of renal failure, more general processes related to chronic kidney disease or compensatory change. (4) The underlying genotype in most participants was not known. (5) It would be interesting to compare the prediction ability of the potential examined biomarkers to predict ADPKD progression within the same papulation, however, with a small number of overlapping patients between the examined biomarkers (**Figure 6.1**), this project could not provide a comprehensive statistically analysis of these biomarkers with only 7 patients. (6) All study participants were Caucasian and therefore the results may not be applicable to other ethnic groups.

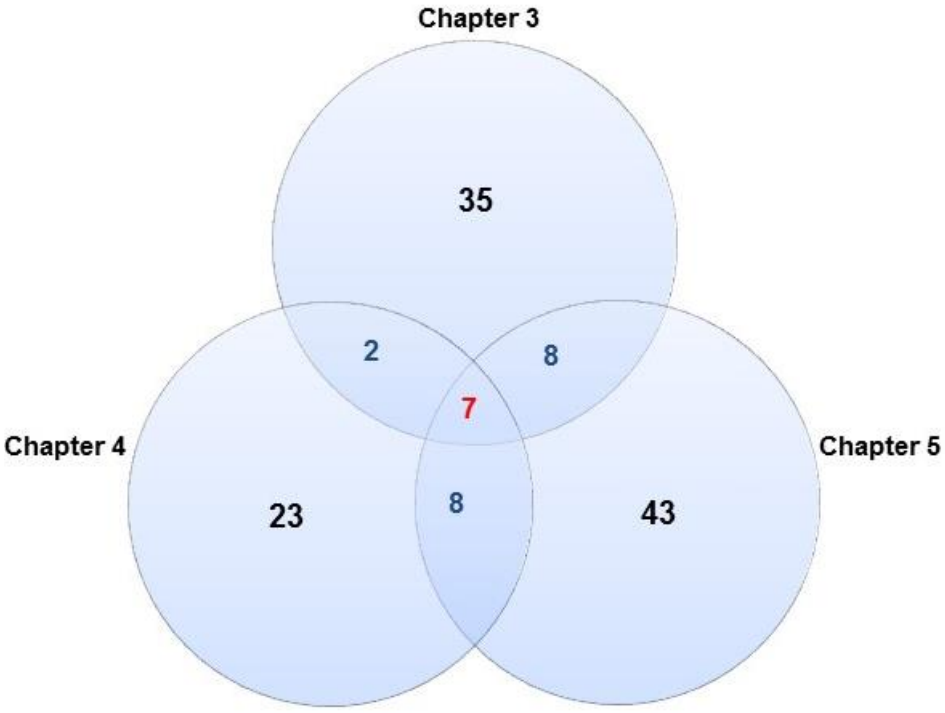

Figure 6.1. Number of patients participated in each chapter

Venn diagram shows common and unique patients participated in the chapter 3, 4 and 5 Red line shows the number of patients participated in the 3 chapters, microRNAs identified by the 3 methods. Blue lines indicate the number pf patients participated in 2 chapters. black lines show the number pf patients participated in a single chapter.

Future directions

These results will need to be validated in another cohort ideally with a long follow-up period to correlate serial changes with clinical measures of disease progression. Development of an easier but efficient technique to isolate urinary exosomes will enable candidate proteins and microRNAs to be validated as new clinical biomarkers. A re-examination of the sequenced reads from this study using different software (miRDeep) could identify additional novel microRNAs. The renal expression of differentially expressed microRNAs could be evaluated next in disease models to correlate changes between renal expression and changes in urinary exosomes. Experimental studies to verify the key predicted target genes regulated by the differentially expressed microRNAs and their functional consequences in cystogenesis should be undertaken. Finally, a number of other angiogenic factors identified by the discovery arrays but not validated in this study could be investigated.

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