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AN INVESTIGATION INTO ENDOTHELIAL DYSFUNCTION IN PATIENTS WITH AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE

BY

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Dedication

This thesis is dedicated

To

My family

This project would not have been possible without their understanding, prayers and support.

Thank you.

Acknowledgements

I give all the glory to the 'I am that I am'. The beginning and the end, God almighty. To Him alone be glory and praise.

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACEi	Angiotensin converting enzyme inhibitor
ADMA	Asymmetric dimethyl arginine
ADPKD	Autosomal dominant polycystic kidney disease
AI	Augmentation index
ARBs	Angiotensin receptor blockers
BMI	Body mass index
BSA	Bovine serum albumin
CFU	Cell free urine
CKD	Chronic kidney disease
CRF	Clinical Research Facility
CRISP	Consortium for Radiologic Imaging Studies of Polycystic Kidney Disease
CU	Complete urine
CV	Cardiovascular
CVD	Cardiovascular disease
DDAH	Dimethylarginine dimethylaminohydrolase
DBP	Diastolic blood pressure
ECM	Extra cellular matrix
ED	Endothelial dysfunction
ELISA	Enzyme linked immunosorbent assay
FMD	Flow mediated dilation
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
cGMP	Cyclic guanosine monophosphate
GP	Glycoprotein
HALT-PKD	HALT Progression of Polycystic Kidney Disease
HO	Heme oxygenase
HV	Healthy volunteer
IHD	Ischemic heart disease

ICA	Intracranial artery
IL	Interleukin
LDL	Low density lipoprotein
LnRHI	Natural log of reactive hyperemic index
MAP	Mean arterial blood pressure
MMP	Matrix metalloproteinase
MSU	Mid-stream urine
NIDDM	Non-insulin dependent diabetes mellitus
NKF/KDOQI	National Kidney Foundation/ Kidney Disease Outcomes Quality Initiative
NO	Nitric oxide
NOS	Nitric oxide synthase
NOx	Total nitric oxide
OCKD	Other forms of chronic kidney disease (Non-ADPKD CKD)
OHdG	Hydroxyl deoxyguanosine
PAT	Pulse arterial tonometry
PBS	Phosphate buffered saline
PCR	Protein creatinine ratio
PG	Prostaglandin
PKC	Protein kinase C
PKD	Polycystic kidney disease
PTH	Parathyroid hormone
PVD	Peripheral vascular disease
RAAS	Renin-angiotensin-aldosterone-system
REJ	Receptor for egg jelly
RHI	Reactive hyperaemia index
ROS	Reactive oxygen species
RRT	Renal replacement therapy
SBP	Systolic blood pressure
SOD	Super oxide dismutase
T2DM	Type 2 diabetes mellitus
TETFUND	Tertiary Education Trust Fund

TIMP	Tissue inhibitor of metalloproteinases
UKRR	United Kingdom Renal Registry
VSMC	Vascular smooth muscle cell
VEGF	Vascular endothelial growth factor
WHO	World Health organisation

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Abstract

Introduction: Cardiovascular complications are the leading cause of death in patients with autosomal dominant polycystic kidney disease (ADPKD) and there is a high incidence of hypertension in ADPKD patients with early disease. Endothelial dysfunction (ED) is an early marker of cardiovascular complications and this has also been reported in ADPKD. However, published studies have included a significant number of patients with later stage disease and other known comorbidities that are also associated with ED including hypertension. The major aim of this thesis was therefore to test the hypothesis that ED was present in ADPKD patients with early disease.

Method: A cross-sectional study was first performed using retrospective serum samples obtained from 60 ADPKD patients, 40 patients with other forms of chronic kidney disease (OCKD) and 36 healthy volunteers. In a subsequent prospective study, 20 ADPKD patients with eGFR ≥60ml/min/1.73m^2 attending the PKD clinic at the Sheffield Kidney Institute were recruited. Exclusion criteria included hypertension, current smokers, age above 50 years, high body mass index (>30kg/m^2) and concurrent use of medications known to be associated with ED. A total of 20 age, sex and eGFR matched healthy volunteers were also recruited. Endothelial function was assessed non-invasively by reactive hyperaemia index (RHI). Serum concentrations of asymmetric dimethyl arginine (ADMA), matrix metalloproteinase 2, and nitrite/nitrate were measured as markers of ED while 8 isoprostane and PGE2 were measured as markers of oxidative stress. Blood was collected for routine biochemistry and urine for 24hr protein and creatinine measurements. Finally, tissue quantification of oxidative stress was assessed by immunohistochemistry in Pkd2 heterozygous mice subjected to ischemia reperfusion injury (IRI) to mesenteric vessels (detecting β hydroxyl deoxyguanosine, β OHdG

protein) or kidney (detecting matrix metalloproteinase 2, MMP2, β OHdG, superoxide dismutase 2, SOD2 and heme oxygenase 1, HO-1 proteins).

Results: In the initial retrospective cross-sectional study, markers of oxidative stress (serum 8 isoprostane) and ED (serum ADMA) were significantly higher in ADPKD patients than in HV but were similarly elevated in patients with OCKD. In the prospective study, there was a significant difference in diastolic blood pressure (DBP) and proteinuria between PKD and HV groups despite the absence of diagnosed hypertension or difference in eGFR ($p<0.001$). RHI was not significantly different between PKD patients and controls. Similarly, no difference in 8 isoprostane, ADMA, MMP2 and PGE2 were found. Conversely, serum nitrite/nitrate was lower in ADPKD patients than in controls. Immunohistochemistry of mesenteric vessels and kidneys of Pkd2 heterozygous mice subjected to IRI revealed greater oxidative stress compared to wild type mice.

Conclusions: Although baseline endothelial function was normal as measured by RHI and some serum biomarkers in ADPKD patients with early disease (eGFR>60ml/min/1.73m^2), the presence of increased proteinuria, lowered serum nitrite/nitrate concentrations correlating with higher DBP suggest some features of ED may be present in early PKD. The detection of similar increases in ADMA and 8-isoprostane in ADPKD with late disease (eGFR<60ml/min) and OCKD suggests similar mechanisms for ED and oxidative stress in both groups rather than a PKD specific pathway of ED. Finally, Pkd2 heterozygous animals appear to be more sensitive to oxidative stress experimentally, suggesting a genetic predisposition to oxidant injury which may be relevant to ED in human disease.

CHAPTER ONE

1.1 Introduction

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a hereditary cause of kidney disease characterised by the development of cysts in the kidneys and other parts of the body such as the liver, arachnoid membrane, pancreas and spleen (1, 2).

ADPKD results from mutation in PKD1 (1) or PKD2 (3). PKD1 is located on the short arm of chromosome 16 position 13 (4) and its mutation is responsible for about 80-85% of ADPKD. PKD2 is located on the long arm of chromosome 4 at position 21 (3) and its mutation is responsible for 10-15% of ADPKD. However, reports from North America showed higher prevalence figures for PKD2, 26% in Canada and 36% in United States of America. (5). These two genes encode for polycystin 1 and 2 respectively. It is controversial whether a third polycystin exists. The existence of "PKD3" was based on the diagnosis of a non-PKD1, non-PKD2 variant of the disease (6-8). However, in a recent study, Paul and colleagues re-evaluated families initially labelled as having PKD3 mutation and concluded there was no evidence for such a gene (9). Individuals with PKD2 mutation have milder disease and delayed onset of hypertension. They also have fewer cysts and there is a delay of about two decades in the onset of end stage renal disease (ESRD) compared with PKD1 mutations (10, 11). However, there is emerging evidence of a milder disease variant of PKD1 associated with non-truncating mutations (12-15).

Polycystin 1 consists of a large extracellular part, 11 transmembrane domains and an intracellular domain (16). It is a member of a novel class of proteins with components whose structures and functions are described in Table 1.1. Polycystin 1 plays a significant role in intracellular calcium homeostasis (17) and membrane trafficking (18). Its involvement in

membrane trafficking explains why its mutation results in mispolarisation of the membrane (19). However, Polycystin 2 is a smaller protein with no extracellular domain. It has 6 transmembrane domains (Table 1.1) and it functions as a calcium channel (20). The Polycystin complex (Figure 1.1) has been localised in the primary cilia where it mediates calcium trafficking in response to mechanical stimulation (21) and this has been described as one of the pathogenetic mechanisms of ADPKD (22).

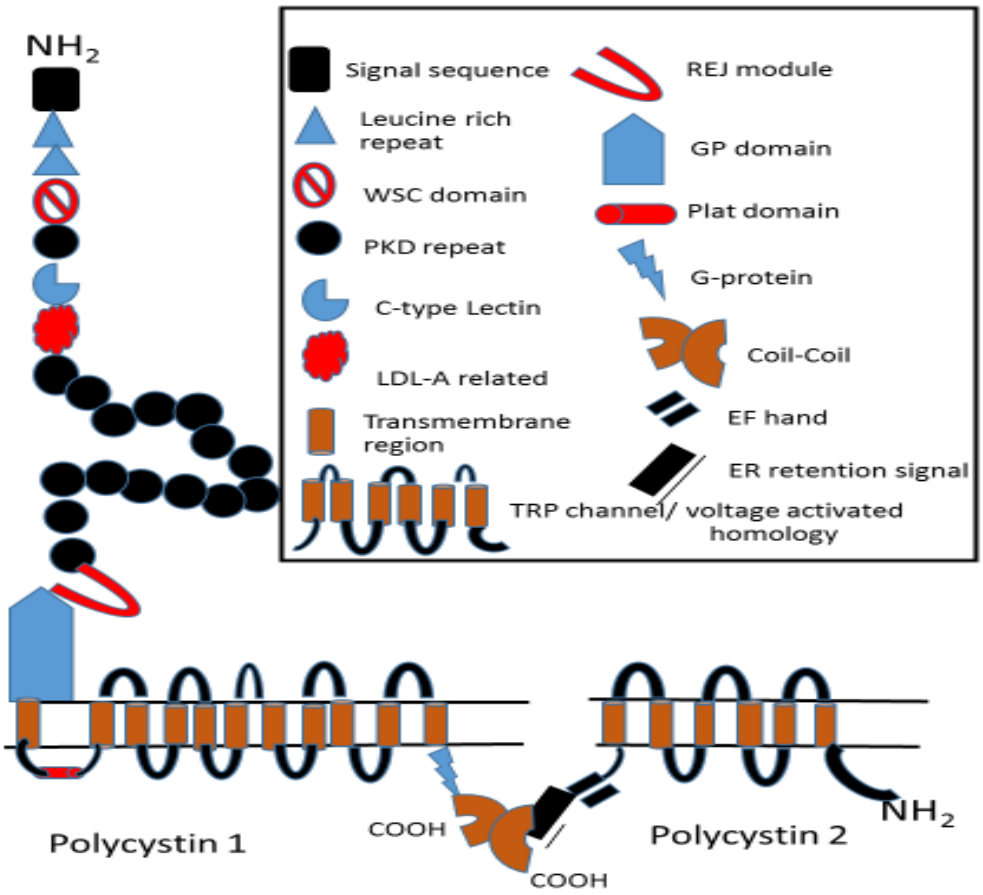


Figure 1.1. Schematic diagram of polycystin 1 and 2.

Table 1.1 Characteristic and function of the various structural motifs of the polycystin complex.

Polycystin 1			
Components	Characteristics	Functions	References
Extracellular N terminal			
Leucine rich repeat	Cysteine flanked	Associated with protein-protein interaction involve in adhesion	(23)
C type Lectin		Predict protein-carbohydrate interaction. Calcium sensitivity	(24)
LDL receptor like protein	40 amino acid domains. Cysteine rich Hydrophobic	Ligand binding	(24)
PKD 1 domain	16 copies. Ig-like	Ligand binding	(25)
REJ module (Receptor for egg jelly)	1000 amino acid sequence.	Involved in calcium influx	(19)
Transmembrane domain			
11 transmembrane regions		Signal transduction	(19)
Intracellular domain			
PEST sequence			
Coil-coil structure	First identified in fibrous protein by Pauling and Cory in 1953	Membrane protein trafficking	(18)
Polycystin 2			
6 transmembrane spanning domains	Homologous to the last 6 domains of polycystin 1 (PC1) transmembrane protein Both N and C terminal are intracellular	Calcium binding via ? EF hand motif in the C terminal ? Transient receptor potential 1 (TRCP 1) channel	(3, 20)

1.2 Epidemiology

The global prevalence of ADPKD is 1:400 - 1:1000 (26). Although the disease affects all races in every part of the world, there is a wide variation in the prevalence figure from one region to another. There are about 12.5 million people with ADPKD worldwide (27) In Europe, Dalgaard reported a prevalence of 1:1000 in Copenhagen (28) and a similar figure was reported by Simon and colleagues in France (29). There are countries with lower prevalence figures such as Wales with 1:2459 (30) and Japan, 1:4033 (31)

The disease is suggested to be rarer in blacks. However, a recent hospital-based study from West Africa reported a hospital prevalence of 18:1000. (32). Similarly, a study from Senegal, West Africa reported a prevalence of 1:280 and concluded that the disease is not rarer in blacks and may be underdiagnosed (33).

ADPKD is commoner than the combination of cystic fibrosis, sickle cell anaemia, muscular dystrophy, Huntington's disease and haemophilia(34) and accounts for 8-10% of patients on renal replacement therapy (35).

1.3 Renal and extrarenal manifestations of ADPKD

ADPKD is often silent in the early phase (30). However, there are documented renal and extra renal manifestations of the disease and these mostly begin by the 3rd to 4th decade of life (36).

1.3.1. **Renal Manifestations of ADPKD**

Kidney enlargement is one way in which ADPKD may present. This is a physical reflection of cyst formation and subsequent cyst growth. Mostly, this is detected during routine screening of individuals at risk and ultrasound remains the most widely acceptable radiological means of cyst detection (37). Cysts can be detected at any age but the chances of diagnosis using

ultrasound increase from the age of 30 years. If ultrasound scanning does not detect cysts at this age, it does not exclude the disease. Conversely, there is a possibility of detection of kidney cyst in ADPKD patients below the age of 30 years (37).

Likewise, Pei and colleagues assessed at risk individuals from both PKD1 and PKD2 families to determine if the current ultrasound diagnostic criteria, which predicts diagnosis based on finding from individuals at risk of PKD was sufficient for both groups(38). They discovered that many at risk individuals were being left out using the current criteria and recommended a unified criterion which will be more useful in areas where molecular diagnosis is seldom carried out.

The Consortium for Radiologic Imaging Studies of Polycystic Kidney Disease (CRISP) is a longitudinal observational study of 241 ADPKD patients that had no features of azotaemia (39). Over three years, the study showed that cyst volume at baseline predicted the rate of renal function decline in those with baseline Total Kidney Volume (TKV) greater than 1500ml. In addition, the rate at which the cysts enlarge strongly correlates with TKV. The average rate of TKV increase is 5.3% per year (39, 40). In a recent single centre study of natural history of ADPKD the mean kidney length was found to be a predictor of renal function, eGFR, in 88 patients being followed up in a dedicated PKD clinic at Sheffield (41)

In another study, Chapman and colleagues assessed the relationship between changes in kidney volume and onset of renal insufficiency in ADPKD patients by utilizing a patient's height corrected determinant of volume changes, height adjusted total kidney volume (htTKV), as against TKV (42). They followed up 241 ADPKD patients with GFR of >70ml/min/1.73m^2 for 7.9 years with the primary endpoint being the attainment of GFR < 60ml/min/ 1.73m^2 during

the study period. They demonstrated that htTKV value of 600cm^3/m was a sensitive predictor of development of renal insufficiency in adult ADPKD patients (42).

Patients with ADPKD may present with haematuria. Laleye and colleagues reported a prevalence of 46% in the cohort of patients from a centre in West Africa (32). Thong and Ong reported a prevalence of 11% and that hematuria was more often the initial presentation in males than in females (41). The onset of haematuria in ADPKD may be the result of an associated kidney stone, urinary tract infection or cyst haemorrhage, where the cyst communicates with the collecting duct.

Other renal manifestations include reduced urine concentration and defective handling of ammonia which could be one of the mechanisms of kidney stone formation in ADPKD patients (43). Common types of stones in ADPKD patients are uric acid, calcium oxalate or both and the treatment is the same as for individuals without ADPKD (44). Loin pain is one of the most frequent symptoms in ADPKD patients, especially in women (41)

1.3.2. **Extrarenal manifestations of ADPKD**

Polycystic liver disease (PLD) is the most common extra renal manifestation of ADPKD (45). Using magnetic resonance imaging (MRI), the CRISP study described the prevalence of liver cysts in ADPKD per age group at diagnosis. The prevalence in those within the age group of 35 to 46 years was 94% while the prevalence is 55% in those younger than 35 years (46). In another study, a prevalence of 55.8% was reported by ultrasound (41). However, the overall prevalence could be as high as 83% such as in CRISP cohort (46). Liver cysts are more common in females (47, 48) and more so in pregnant women and women with a history of contraceptive use (2). These underpin the role of oestrogens in the pathogenesis of this disorder. Binding of estrogen to receptors on cholangiocytes stimulate the release of growth

factors leading to cell proliferation (49). Lee and colleagues described the role of microRNAs in the development of liver cysts in rat model of ADPKD (50). They reported an association between decrease in miR15a expression and upregulation of its target, the cell cycle regulator cell division cycle 25A (Cdc25A). This association suggested that miR15a contributes to hepatic cytogenesis through dysregulation of Cdc25A.

PLD is usually asymptomatic but can manifest acutely as cyst haemorrhage or infection. Cyst infection may present as fever and right upper abdominal pain and the diagnosis or localization of the disease process can be difficult (51). However, Sallee and colleagues described the use of positron emission tomography (PET) scanning in 4 patients with hepatic cyst infection and suggested that this method of diagnosis is promising as they were all scan positive (52). The major limitation of PET scan is that it is less discriminatory as it sometimes shows up areas of high activities. It is also not freely available(53).

Although Gabow and Grantham had suggested that there were no known spinal manifestations of ADPKD (54), Schievink and Torres convincingly described three cases of spinal meningeal diverticula in a cohort of 178 ADPKD patients who were screened for intracranial aneurysms (55). Therefore, the diagnosis of spinal meningeal diverticula should be considered in ADPKD patients presenting with new onset headache.

Concerning extra renal vascular association, the commonest finding is intracranial aneurysms. (ICA) (56). Pirson and colleagues reported a prevalence of 8% (57) while Rinkel suggested a prevalence of 2% (58). Thong and Ong reported a prevalence of 4.8% in their survey (41). However, the prevalence varies widely between 4% to 41.2% (59-61). The variability depends on study population. Population with family history of intracranial aneurysms or

haemorrhagic stroke has higher prevalence than those without these risk factors. The prevalence is even higher in older age groups(62).

Although most ICA are asymptomatic (57), the most common symptom preceding rupture is headache. This was reported in 38% of patients who were originally admitted on suspicion of subarachnoid haemorrhage (SAH) (63). ICA are more likely to rupture in females in the general population (64). However, no gender difference has been reported in the rate of ICA rupture in patients with ADPKD (63, 65). This observation has led to the suggestion that estrogen might not play a significant role in the pathogenesis of ICA rupture in ADPKD (66). More work is required in this area. The average age at which ICA ruptures in ADPKD patients, 41 years, is less than that of the general population, 51 years (65).

In addition, a disorder of elongation and dilatation of segments of intracranial vessel known as dolichoectasia was described in 2% of ADPKD patients in whom it was found to be a cause of cerebrovascular accidents (67).

1.4 **Cardiovascular abnormalities in ADPKD**.

Cardiovascular events are the commonest cause of death in patients with ADPKD (68). Spithoven and colleagues reviewed prevalence and analysed data from the ERA-EDTA registry to ascertain the pattern of renal replacement therapy for ADPKD patients in Europe over two decades. They reported cardiovascular events as the leading cause of mortality in ADPKD patients as well as patients with non ADPKD chronic kidney disease (27). Similarly, there is likely to be a direct role of the mutations of PKD1 and PKD2 in the vascular complications of ADPKD. This suggestion followed the detection of strong polycystin-1(69) and polycystin-2 (70) expression in arterial vascular smooth muscle cells (VSMC) with altered pattern of expression in vascular aneurysms. Different types of cardiovascular events have been

associated with ADPKD and the commonest remains hypertension, present in 50-70% of patients without evidence of renal impairment (71, 72). Hypertension occurs at an earlier age in ADPKD compared with the general population (73). Ozakok and colleague followed 323 ADPKD patients for over nine years and reported that hypertension correlated strongly with rate of decline in GFR. They concluded that hypertension is one of the two major treatable surrogate markers for disease progression, the other being proteinuria (74). Similarly, Helal and colleagues in a recent review, reported that 86.6% of 426 ADPKD patients were hypertensive with a mean age at diagnosis of 36.9±12.9 years and 32.5% had progressed to ESRD (75). Other CV complications include left ventricular hypertrophy, found in 48% of hypertensive ADPKD (76). This was higher than that seen in unselected essential hypertensive patients (12 -30%) but similar to the average described in hypertensive patients seen in referral centres (20-60%) (77-79). In a recent study Pietrzak-Nowacka found an association between left ventricular mass index and hypertension in female ADPKD patients but not in sex matched controls (80).

Similarly, cardiac valve defects have been described in ADPKD and often present as mitral valve prolapse (MVP) and mitral regurgitation. This is a common presentation and the prevalence is about 25% (81). Gabow and colleagues assessed various factors associated with progression of renal disease in 580 ADPKD patients and concluded that mitral valve prolapse is not one of them. (82). Most often, there is no need for valve replacement (2).

1.5 **Pathogenesis of hypertension in ADPKD**.

Cyst expansion has been postulated to disrupt kidney parenchymal architecture leading to activation of the renin-angiotensin-aldosterone system with a resultant increase in blood pressure (83, 84). Sympathetic hyperactivity has also been implicated as a contributory

factor to the development of hypertension in ADPKD (85) and may increase CV risk irrespective of blood pressure (86). Klein and Colleagues in a study of ADPKD patients with normal and abnormal renal function concluded that muscle sympathetic nerve activity (MSNA) is increased in hypertensive ADPKD irrespective of renal function (85). This study utilised MSNA unlike previous studies where adrenaline was used to determine sympathetic activity in animal models of hypertensive kidney failure (87, 88). Some papers have described MSNA as a better predictor of sympathetic nerve activity in hypertension (89). Sympathetic nerve activity increase can lead to hypertension in ADPKD patients. Enlarging cysts produce distortion of the renal parenchyma which may result in renal ischemia with attendant disordered sodium balance or increase renal afferent nerve activity.

1.6 **Current treatment modalities of autosomal dominant polycystic kidney disease.**

There have been reports of significant progress made over recent years in the area of treatment of hypertension in ADPKD and of the primary disease itself (90, 91). Schrier and colleagues reported a decrease in kidney disease progression in ADPKD patients with better blood pressure control using angiotensin converting enzyme inhibitors. (90). However, the recently concluded HALT-PKD study reported that there was no significant difference in kidney disease progression between ADPKD patients in whom angiotensin converting enzyme inhibitors alone were used and those with dual blockade of renin angiotensin aldosterone system (92). Apart from blood pressure control, other treatment modalities are being developed to slow disease progression. The most significant of these is the use of V2 receptor antagonist which worked impressively in animal models (93, 94). A clinical trial in patients with ADPKD using the V2 receptor antagonist, Tolvaptan, showed that the drug produced a

remarkable slowing down of increase TKV and reduction in rate of kidney function decline as compared to placebo. However, higher discontinuation rate was reported in this study due to adverse events effects which were related to increase aquaresis (polyuria, nocturia, thirst and polydipsia) in the patients who received tolvaptan (95). Essentially, V2 receptor antagonist blocks cAMP by acting on V2 receptors at the distal nephron and the collecting duct (96). cAMP enhances cyst growth and is over expressed in patients with ADPKD (94). Tolvaptan was recently licensed for use by European Medicine Agency in ADPKD patients with early disease (CKD stages 1-3) at initiation of treatment with evidence of rapid disease progression or TKV size (97). A study of effect of tolvaptan on later-stage ADPKD known as Replicating Evidence of Preserved Renal Function: An Investigation of Tolvaptan Safety and Efficacy in ADPKD (REPRISE study) was recently concluded. The primary end point in that study was the change in eGFR from baseline to follow up to post treatment follow up. This change was -2.3ml/min/1.73m^2/year with tolvaptan versus -3.6ml/min/1.73m^2/year with placebo, corresponding to a treatment effect of 1.3ml/min/1.73m^2/year, which was statistically significant. Torres and colleagues concluded that tolvaptan resulted in slower decline in eGFR than placebo over a period of 1 year in later-stage ADPKD patients(98). After the REPRISE study, the United States Food and Drug Administration (FDA) approved the use of tolvaptan as first drug treatment to slow kidney function decline in ADPKD patients at risk of rapidly progressing disease (99).

Similarly, increased fluid intake has been postulated to be beneficial in patients with ADPKD by suppressing vasopressin secretion (100, 101).

1.7 Endothelium: structure, function and dysfunction.

The endothelium comprises of an innermost layer of endothelial cells, a middle layer of glycocalyx and an outermost layer of basement membrane (102). The arterial endothelium (Figure 1.2) has been demonstrated to be different from that of the vein in terms of embryonic origin (103). Similarly, Adams and Alitalo described the different behaviours of the vascular beds in terms of their tissue specific characteristics and functions (104). The arterial vascular endothelial cell can generate vasodilators and vasoconstrictors (105). Example of endothelium-secreted vasodilators are prostacyclin and nitric oxide. It can also produce vasoconstrictors, endothelium derived contractile factors (EDCF), like thromboxane A2, (106). Its ability to secrete vasodilator substances and regulate arterial tone underlies its involvement in blood pressure regulation (107).

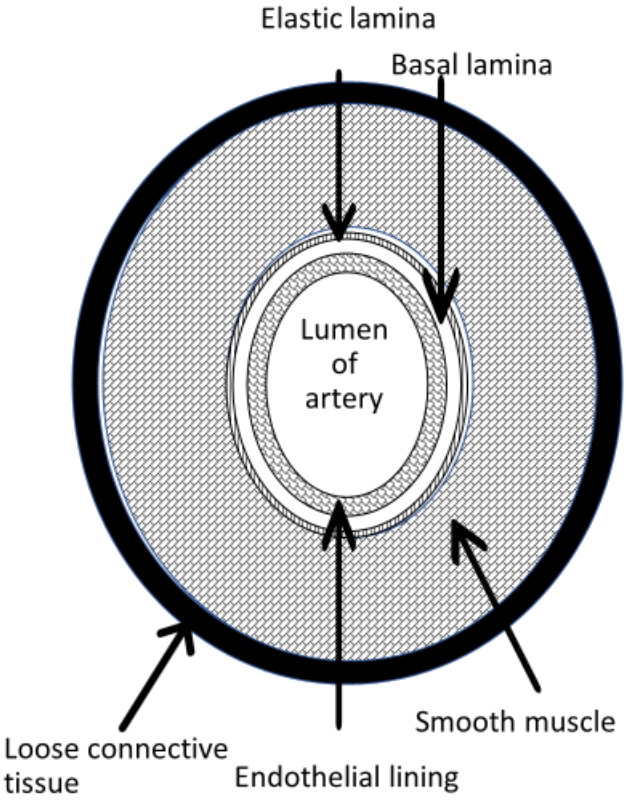


Figure 1.2: Cross section of a small artery showing the location of the endothelium.

The endothelium regulates vascular tone by production of nitric oxide from L-arginine. It also functions to prevent platelet aggregation and blood clotting (108), neutrophil recruitment and hormone trafficking. The endothelium is the main barrier between blood and tissue (106). Its permeability to a molecule depends partly on the size and charge of the molecule. When its permeability increases, tissue oedema results. Similarly, it contains heparan sulphate, a co-factor for activating anti thrombin which inhibits several clotting factors (108).

Endothelial dysfunction (ED) can be defined as reduced bio-availability of Nitric Oxide (NO) which plays many roles in maintaining vascular health, especially its role in vasomotion. Hence, ED is defined as an impairment of endothelium dependent vasodilation(109).

Furthermore, the endothelium takes part in angiogenesis (110). Vascular endothelial growth factor (VEGF) is produced by several cell types and acts selectively on endothelial cells. Apart from maintaining the integrity of endothelial cells, the role of VEGF in angiogenesis has been well described. There is a connection between VEGF and ED based on the VEGF receptor 1, also known as soluble fms-like tyrosine kinase-1 (sFIt-1) produced by endothelial cells, placenta and monocytes (111). SFIt-1 is a potent antagonist of VEGF action. Hornig et al concluded that release of sFIt-1 from the endothelial cell (and from other biological fluid) is associated with significant ED (112).

Furchgott's experiments on the endothelium opened the gateway to the understanding of the role of NO-dependent endothelial relaxation (113). He stripped off the endothelium from arterial preparations, thus preventing it from responding to acetylcholine stimulation. However, not all arteries are innervated by cholinergic fibres and those with cholinergic fibres have their neurons in the adventitial of the vessel (114). This makes the response more pharmacologic than physiologic (114). There are more physiologic stimuli which include shear or physical stress. Other physiological mediators include serotonin, arginine vasopressin, aldosterone, histamine and bradykinin (115, 116).

ED occurs when the balance between NO induced vascular relaxation and the vasoconstriction produced by endothelium derived contracting factor (EDCF) is altered (114). Nitric oxide release is enhanced by dietary factors, exercise and oestrogen. Conversely, it is

blunted by events such as smoking, oxidative stress (117) and oxidation of low density lipoprotein (114).

Brunner and colleagues summarised the various mechanisms through which reactive oxygen species (ROS) cause endothelial dysfunction (118). These include direct cellular damage, mopping up of nitric oxide to form peroxynitrite, a potent oxidant. ROS also have indirect effects on lipid peroxidation (119).

Endothelial derived nitric oxide synthase (eNOS) converts L-arginine to nitric oxide following the release of the former from the endothelium as a result of shear stress. Asymmetric dimethylarginine arginine (ADMA) is known to inhibit eNOS activity and is a very important biomarker of cardiovascular risk (120). Also, dimethyl arginine dimethylamino hydrolase (DDAH) breaks down ADMA before its excretion by the kidney (121). DDAH activity is inhibited by oxygen free radicals which themselves are converted to hydrogen peroxide by the actions of superoxide dismutase (SOD) and heme oxygenase 1 (HO1). Nitric oxide moves into the vascular smooth muscle where it activates guanylate cyclase, an enzyme required for smooth muscle relaxation (Figure 1.3).

The smooth muscle relaxation effect of cGMP can be inhibited by phosphodiesterase 5.

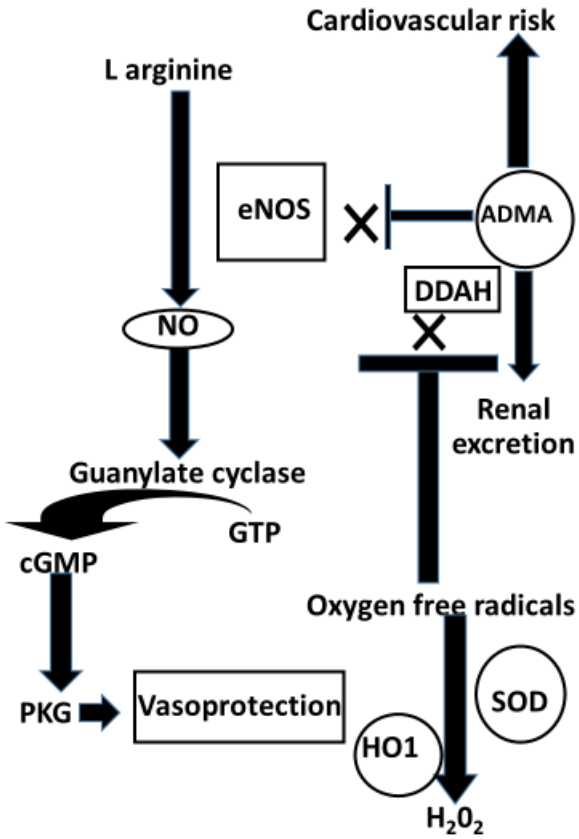


Figure 1.3: Nitric oxide formation and effects.

1.8 Factor affecting endothelial function.

This is best categorised either into factors that up-regulate or down-regulate NO release from the endothelium (114).

Factors that upregulate NO dependent endothelial relaxation include shear stress, oestrogen, insulin, adiponectin, testosterone, thyroid hormone, glucagon like peptide, arginine, dietary agents like ω_3 unsaturated fatty acid (122), flavonoids (123) and other polyphenols as found in red wine (124), green tea (125), grape juice (126) and dark chocolate (127).

Conversely, factors that down regulate NO dependent endothelial relaxation include oxygen derived free radicals (oxidative stress). This results in peroxynitrite production which leads to

decreased bioavailability of NO (128). Other factors that reduce NO production include chronic aldosterone administration (129), ageing (130), smoking (131), homocysteinemia (132), hypercholesterolemia (133), obesity (134) and sleep apnoea (135).

1.9 Assessment of endothelial dysfunction

1.9.1 Non-invasive assessment of endothelial dysfunction.

Flow mediated dilatation: This is mainly performed by assessing the effect of shear stress on the endothelium. Rubanyi et al postulated that shear stress up regulates NOS activity in a Ca^{2+} independent manner, resulting in enhancement of NO release and promotion of smooth muscle relaxation (136). This is the basis of the use of flow mediated dilation (FMD) in assessing endothelial function (114). Celermajer and colleagues were the first to use ultrasound to measure FMD. They assessed ED in symptom-free young adults and children at risk of atherosclerosis using high-resolution ultrasound. This was done by measuring the diameter of brachial and superficial femoral arteries at rest, following sublingual administration of glyceryl trinitrate (GTN) and during reactive hyperemia (137). Subsequently, Ioannides et al demonstrated that this method of FMD was NO dependent (138, 139). It has been described as the Gold standard method of non-invasive assessment of ED (140) and reported to predict cardiovascular events (141). Also, this method has been used in participants without heart disease (142) and in community based studies (143). However, there are limitations regarding the use of ultrasound to assess flow mediated changes in the brachial artery. It is significantly operator dependent (144) and this is despite efforts by the International Brachial Artery Reactivity Task Force to produce guidelines for effective use of this technique (145). Also, it is technically challenging and requires adequate training. Other limitations include reported attenuation of signals in obese subjects (146) and low

reproducibility (141). Reports in the literature regarding the use of vascular ultrasound to assess FMD (FMD-VUSS) in early disease ADPKD patients are sparse and those available differ in their conclusions. While some groups have reported impaired FMD (147, 148), Clausen and colleagues found no difference between patients and healthy controls (149).

Venous occlusive plethysmography: This is a semi-invasive method of assessing ED. It involves cannulation of brachial artery through which acetylcholine or nitroglycerine can be infused for endothelial dependent and non-endothelial dependent dilatation respectively (150, 151). Novel substances, agonists and antagonists can also be administered slowly through the brachial artery to test their effects. The contralateral limb serves as a control. Blood flow changes produced by these agents can be measured by plethysmography in both arms. Results are expressed as the ratio of blood flow in both the test and contralateral arms.

Finger plethysmography: The limitation of observer dependency has made many endothelial researchers to opt for digital pulse amplitude tonometry, EndoPAT (152). This method works by assessing the plethysmographic changes in pressure caused by arterial pulsations at the fingertip (153). Fingertip pressure changes have been reported to correlate with coronary microvascular function in individuals with early atherosclerosis, with traditional as well as non-traditional risk factors of cardiovascular disease (154, 155). This newer non-invasive method of assessing ED has been reviewed in the literature (152, 156, 157). Just like FMD, a pressure cuff is used on the test arm while the contralateral arm serves as control. Tamler and Bar-Chama, while comparing methods of non-invasive assessment of ED concluded that EndoPAT predicts cardiovascular events beyond the Framingham risk score (156). Similarly, EndoPAT identified patients at risk of cardiovascular events even when traditional Framingham risk score failed to identify the same (158). This method has also been used to

assess response to therapy (159) and has been approved by the United States Food and Drug Administration (FDA) (154). However, there are also reports concluding that no correlations exist between FMD as measured by brachial ultrasound and RHI as measured by finger plethysmography (160, 161). Similarly, Lind evaluated the relationship between methods of ED assessment and concluded that RHI had no correlation with acetylcholine mediated vasodilatation and ultrasound assessment of FMD (162). These observed differences suggest that there may be another physiological basis responsible for the vascular reactivity in finger plethysmography (163). It has been reported to be only partly dependent of NO as it evaluates small vessel dilatation. However, Nohria et al investigated the role of NO in the vascular reactivity in finger plethysmography by inhibiting the eNOS with nitro-L-arginine methyl (L(NAME) in nineteen healthy volunteers and compared the effect with that observed following administration of saline and phenylephrine, a nonspecific vasoconstrictor. They reported that NO has a central role in vascular reactivity as measured by finger plethysmography (164).

Pulse wave velocity and pulse waveform analysis: This technique essentially assesses vascular wall stiffness as a parameter known as augmentation index (165) and could be used to measure FMD (166). Aortic pulse wave velocity (PWV) is normally measured between carotid and femoral arteries by synchronically assessing arrival of pulse wave at both locations and calculating the time difference. ED has been reported to play a role in arterial stiffness and assessment of PWV has been used to investigate effects of endothelial mediators (167).

Other non-invasive methods: Other techniques include use of magnetic resonance imaging (MRI)(168, 169).

1.9.2 Biomarkers of endothelial dysfunction and oxidative stress in ADPKD

Several biomarkers have been used to assess ED in ADPKD. Table 1.2 summarises the biomarkers of oxidative stress and ED investigated in this study. Many of these previous studies recruited participants with features that were confounders for oxidative stress and endothelial dysfunction. For instance, Menon and colleagues in their investigation of inflammation, oxidative stress and insulin resistance in ADPKD concluded that inflammation and oxidative stress were evident in ADPKD patients with preserved kidney function , eGFR>60ml/min/1.73m^2(170). However, confounding factors like smoking were not excluded and smoking is known to be strongly associated with oxidative stress (171, 172).

Antioxidant systems can mop up reactive oxygen species (ROS) during normal physiological condition. However, this mopping up process is overwhelmed under disease conditions resulting in lipid, protein and DNA damage (173).

8 -Hydroxydeoxyguanosine (8OHdG)

Nucleobases are oxidised following ROS mediated DNA damage. The most widely recognised product of oxidative DNA damage is 8 hydroxy-2-deoaxyguanosine (8OHdG) which is formed as a result of guanosine oxidation (174, 175) and which has been found to be associated with cardiovascular events (176, 177). However, there is significant disagreement in the reports of 8OHdG association with cardiovascular events and this heterogeneity has recently been linked with variability in the laboratory methods used in the various studies (178). There is paucity of data on 8OHdG immunostaining as a marker of oxidative stress especially in autosomal dominant polycystic kidney disease.

Superoxide Dismutase 2 (SOD2)

Superoxide dismutase 2 (SOD2), also known as manganese-dependent superoxide dismutase (MnSOD) has been reported to have a cardio protective effect which is linked to its ability to mop up ROS (179) as well as its involvement in apoptotic signalling (180). The importance of SOD2 as a novel biomarker of oxidative stress in ADPKD has been reported (170). However, its importance as a marker of cardiovascular events in ADPKD has been underreported.

Heme Oxygenase-1 (HO-1)

Heme oxygenase 1 is a stress inducible enzyme that has been reported to have roles beyond its traditional involvement in heme metabolism. There is an accumulating body of evidence to support its protective role in non-heme-mediated injury like oxidant induced cell injury (181, 182) and there are various explanations for the mechanisms of these protective roles (182). There is growing interest in the search for this enzyme in the area of therapeutic options of cardio protection but this is presently understudied in ADPKD. Heme oxygenase 1 (HO-1) is a marker of oxidative stress and the expression of HO-1 mRNA was reported to be increased in cystic kidney disease rat and mice models (183). Courtney and colleagues reported the association between HO-1 and survival in ADPKD patients and concluded that there was no association between HO-1 gene expression and survival in ADPKD patients (184). However, there is a paucity of report on investigation of HO-1 as biomarker of ED in early disease ADPKD patients.

Matrix Metalloproteinase 2

Matrix metalloproteinase 2 (MMP2) is a member of the matrix metalloproteinase (MMP) family of proteins that are well known for their roles in the breakdown of extracellular matrix (ECM). They have been widely studied in chronic disease processes (185, 186). A recent paper found an association between increased activity of MMP2 and endothelial dysfunction and development of hypertension (187). Matrix metalloproteinase 2 (MMP2) has been reported to be synthesised and secreted by the tubules of ADPKD patients (188). However, there is no report on involvement of this protein in endothelial function in ADPKD patients.

Prostaglandin E2 (PGE2)

Prostaglandins are lipid-derived autacoids (local hormone like biological factors with a brief duration of action which acts near the site of synthesis) involved in mediating vascular tone (189). They are arachidonic acid derivatives. Phospholipases liberate arachidonic acid from membrane phospholipids. This is then metabolized into prostaglandin G (PGG) and prostaglandin H2 (PGH2) by cyclooxygenases-1 (COX-1) and cyclooxygenases (COX-2) with subsequent conversion by prostaglandin E synthase to PGE2 (190, 191). Prostaglandin E2 is known to promote either smooth muscle contraction or relaxation depending on the tissue (192, 193) and its cellular effects are mediated through four E-prostanoid receptors, EP1-4 (194). The balance between pressor and depressor activities of these receptors determines the overall effect of PGE2 on arterial pressure (195). PGE2 is known to promote cyst proliferation and chloride secretion in ADPKD (196). However, PGE2-EP4 receptor signalling has been reported to have anti-inflammatory and cytoprotective properties (197). Also, Kvirkvelia and colleagues observed clinical and pathological improvement in nephritic mice following the administration of PGE2 (198).There has been a single report of increased serum

levels of PGE2 in ADPKD patients with preserved kidney function as compared with healthy controls but participants with ongoing use of medications and other confounders were recruited into that study (199). There is a need to investigate PGE2 as a biomarker of oxidative stress in normotensive early disease ADPKD patients devoid of features that are known risk factors for oxidative stress.

Asymmetric Dimethylarginine (ADMA)

This is an endogenous inhibitor of NO-synthase formed during proteolysis of methylated proteins. It is either excreted by the kidney or metabolically degraded by dimethylarginine dimethylaminohydrolase (DDAH) in a pathway that has been reported to be critical in regulating NO dependent vascular homeostasis (200). An increase in plasma concentration of ADMA was reported in 27 patients with early ADPKD by Wang and colleagues (201). However, confounding factors like smoking were not excluded in the selection of participants in that study.

Nitrate/nitrite and total nitric oxide (NOx)

Nitric oxide is a pleiotropic (able to produce more than one effect) biological mediator with potent vasodilating properties (202). Endothelial dysfunction can result from accelerated degradation of nitric oxide (NO) by ROS (150). It is a gaseous free radical with a short half- life *in vivo* but there are various studies reporting measurement of more stable NO metabolites, nitrite/nitrate (203, 204) and their association with ED (205, 206). Reduction in nitric oxide levels has been reported to predispose to hypertension, vasospasm and atherosclerosis (207, 208). Also, Wang and colleagues reported reduced levels of NO in 9 normotensive and a more severe reduction in 6 hypertensive ADPKD patients (209). However, there was no reference

to the stages of chronic kidney disease in these 2 groups. There is a paucity of data on the level of NO in early disease ADPKD patient without confounding factors of ED.

Table 1.2: List of biomarkers of oxidative stress and endothelial dysfunction used in this study with reference to what is known about them in ADPKD.

Biomarkers	**Experimental model**	**What is known**	**References**
8 isoprostane	Human	Increased in ADPKD patients	(170, 199)
Asymmetric dimethyl arginine	Human	Increased in ADPKD patients	(199, 206)
8 hydroxyl deoxyguanosine	Human	Increased following oxidative stress in ADPKD patients	(210)
Superoxide dismutase 2	Human	Decreased following oxidative stress in ADPKD patients	(170)
Matrix metalloproteinase 2	Human	Biomarker of ED in other disease conditions. Not previously studied as biomarker of ED in ADPKD	(187, 188)
Heme oxygenase 1	Human, *murine*	Increased in ADPKD patients as well as cystic kidneys of mice and rat	(184), *(183)*
Prostaglandin E2	Human	Increased in ADPKD patients	(199)
Nitrate/nitrite	Human, *murine*	Decreased following ED in ADPKD patients and Pkd2 mice	(205, 206) *(211)*
Highly selective C reactive protein	Human	Correlates with ED in ADPKD patients	(212)

Other related biomarkers not used in this study

There are several other related biomarkers that were not used in this study (Table 1.3). Some of these are discussed below.

Malondialdehyde

Malondialdehyde (MDA) is an organic compound that is produced following lipid peroxidation of polyunsaturated fatty acids. It causes toxic stress in cells and forms covalent protein referred to as advanced lipoxidation end product (213)**.** Tariq and colleagues reported increased level of MDA following oxidative stress in ADPKD patients(214). However, there has been reports of variability in results when MDA was used as biomarker of oxidative stress in different disease conditions (215).

Intercellular adhesion molecules

Intercellular adhesion molecules (ICAMs) as well as vascular cell adhesion molecule-1 (VCAM-1) are parts of the immunoglobulin superfamily. They play significant roles in cellular signalling, immune responses and inflammation. ICAM consists of five members namely ICAM-1,ICAM-2, ICAM-3, ICAM-4 and ICAM-5 (216). ICAM-1 has been reported to be upregulated following oxidative stress in other disease conditions (217, 218). There is paucity of reports of the activity of adhesion molecules in ADPKD patients.

Glutathione

Reduced glutathione (GSH) is an antioxidant that is synthesized in the body. It plays a role in the prevention of damage to cells caused by ROS. GSH can be converted to its oxidized form (GSSH) which is also known as L-(-) glutathione. Conversely, GSSH can be reduced back to GSH by glutathione reductase (219). The ratio of GSH to GSSH is often used as a measure of oxidative stress(220). The antioxidant activity of glutathione has been reported to be decreased in ADPKD patients and suggested to play a role in disease progression(221).

Table 1.3: List of other biomarkers of oxidative stress and endothelial dysfunction not used in this study with reference to what is known about them in ADPKD.

Biomarkers	**Experimental model**	**What is known**	**Reference**
Malondialdehyde	*Murine,* Human	Increased following oxidative stress in ADPKD patients, cystic kidneys of mice and rats	*(183*, 214)
Intercellular adhesion molecule	Human	Increased circulating level in ADPKD patients	(222)
Vascular cell adhesion molecule	Human	Increased circulating level in ADPKD patients	(222)
Reduced glutathione	Human	Decreased activity in ADPKD patients	(221)
Oxidized glutathione	Human	Decreased activity in ADPKD patients	(221)

1.10 Research questions

(i) Is there an increased level of biomarkers associated with endothelial dysfunction in patients with ADPKD with preserved kidney function?

(ii) Does Pulse Arterial Tonometry detect endothelial dysfunction in ADPKD patients with preserved kidney function?

(iii) Is there evidence of oxidative stress in the vasculature or tissue of a murine model of pkd2 loss-of-function?

CHAPTER TWO

MATERIALS AND METHODS

This study was approved by the Research Department of Sheffield Teaching Hospitals NHS Trust (application number STH15983). Copies of patients' information sheet, consent forms, study protocol and approval are attached to this thesis. I conducted the study in line with the declaration of Helsinki for health care research in humans, the international conference on harmonisation and good clinical practice guideline (ICH-GCP) of the European Union (EU).

2.1. A retrospective cross-sectional study of biomarkers of oxidative stress and endothelial dysfunction in ADPKD patients and those with other forms of chronic kidney disease compared with healthy volunteers.

Plasma samples were obtained from a previous cross-sectional study of ADPKD (n=60) at stages 1-4 with a non-ADPKD (n=40) CKD group conducted by Professor Ong. Plasma samples from healthy volunteers (n=36) were obtained from a bank of cardiovascular study volunteers at Sheffield Teaching Hospitals.

2.2 A prospective cross-sectional study of oxidative stress and endothelial dysfunction in early stage disease (CKD1 and 2) ADPKD patients and age, sex and eGFR matched healthy volunteers.

The clinical cohort of participants in the retrospective study had early to late stage chronic kidney disease. To test the hypothesis that there is oxidative stress and ED in ADPKD patients with early disease, I next recruited normotensive ADPKD patients with eGFR ≥60ml/min. The exclusion criteria included age <18 or >50 years, hypertension or the use of antihypertensive medications, diabetes mellitus, use of any regular medication except oral contraceptives, pregnancy, body mass index (BMI) > 35kg/m^2, presence of any musculoskeletal condition that

would contraindicate the inflation of blood pressure cuff to suprasystolic pressure (as part of EndoPAT study). I defined hypertension based on National Institute for Health and Care Excellence (NICE) guideline which defines stage 1 hypertension as clinical blood pressure of 140/90mmHg or higher (223). I excluded smokers (current or ex) from this study. Participants were recruited through posters, post and direct interview at the specialised ADPKD clinic at the Sheffield Teaching Hospital. Those who consented were invited to the NIHR Sheffield CRF at the Royal Hallamshire Hospital for the study. A total of 20 ADPKD patients and 20 age, sex and eGFR matched healthy volunteers were recruited. They were instructed to fast overnight and refrain from drinking coffee. They also provided a 24-hour urine sample after they had been instructed on the proper method of collection. Blood as well as urine samples were taken from them during the visit for routine biochemistry as well as for biomarker studies. To test the hypothesis that storage of samples with antioxidant significantly affects the concentration of biomarkers of endothelial dysfunction, I collected samples into two different sets of containers. One set had butyl hydroxy toluene (BHT) as antioxidant while the other set had no antioxidant. These samples were transferred on ice to the laboratory where they were aliquoted and stored at -80^{0}C.

2.3 Statistical analysis

Data was entered Statistical Package for Social Sciences (IBM SPSS, Inc., Chicago IL; version 22) and Graphpad Prism. Continuous variables were expressed as mean± SD while categorical data were expressed as percentages(proportions). Means of subgroups were compared using parametric t test and Mann-Whitney *U*-test where data was non-normally distributed. Chi square was used to compare the frequency distribution of categorical variables. Associations between different variables was examined using Pearson's correlation coefficient (r).

Univariate and multivariate correlation analysis were used to test the association between variables. All p values were two tailed and $p<0.05$ was considered as being statistically significant.

2.4 EndoPAT for non-invasive assessment of endothelial dysfunction in ADPKD.

I tested the hypothesis that pulse arterial tonometry could detect ED in ADPKD patients with early disease by non-invasive assessment. I used EndoPAT 2000 machine from Itamar Medicals, Israel. The machine was installed on a computer system at the Clinical Research Facility (CRF) at Royal Hallamshire Hospital, Sheffield as instructed by the manufacturer.

All patients and healthy volunteers were consented, and their endothelial function was assessed non-invasively as follows.

Participant preparation: Participants fasted for at least 4 hours and abstained from caffeine containing drinks for at least 8hours before the study. They were advised to wear loosely fitting clothes and be free of jewellery in the upper extremities.

The location of the test was in a quiet and temperature-controlled room with moderate intensity lighting. This was in a dedicated room at CRF at the Royal Hallamshire Hospital in Sheffield. Blood pressure cuff was applied to the upper arm of the patients and they could relax for at least fifteen minutes. Blood pressure was first measured in the arm that was not occluded during the study. The cuff was then moved to the arm that would be occluded.

Preparing the EndoPAT system: The EndoPAT 2000 software on the PC was opened and a new patient file was created by completing the information dialogue box on the screen.

Two new PAT probes were selected and connected to the pneumoelectric tubings by connecting them to the connector tab. This produced a click sound and that served as

evidence of a perfect connection between the tab and the probe. The probe was then inserted into the socket of the arm support. This was followed by deflation of the PAT probe.

Conducting the EndoPAT study: Participant's index finger was selected (making sure it was free from cuts and injuries) and placed all the way to the back of the probe until the rim at the back of the probe was felt. The fingernails were appropriately trimmed when noticed to be long enough to affect contact between the fingertip and the PAT probe.

The probes were then inflated after 5 seconds. The foam ring was attached to the middle fingers and pushed to the very end to make sure that the foam and the probe did not touch. 10cm loop was created with the attached tubing and it was anchored with a tape. Participant's arm was positioned on the arm support and the fingers could dangle freely. Information concerning the discomfort that could arise from the 5-minute inflation of the cuff was given to the participant. This was necessary for further reassurance and calm. The system was put in standby mode while the time base was adjusted to 1 minute. The signal gain was adjusted to maximize the signal quality while the two PAT probes were inspected and the time base adjusted to 1minute. The recording was started and timed. This automatically initiated the 5 minutes' baseline recording period. The occlusion phase recording was preceded by rapid inflation of the cuff to 200mmHg or 60mmHg above the SBP, whichever was greater. The time base was changed to 15 seconds and the signal gain on the occluded arm was changed to 20000 at this stage. The occlusion phase recording was then started and timed accordingly. This was stopped after 5 minutes, and the cuff deflated. Post occlusion phase recording was commenced immediately and was appropriately timed for 5 minutes with the stop clock. The fifteen minutes recording was completed at the end of third 5-minute recording phase and the PAT probes were removed, disconnected and discarded.

The data generated during the recording session was reviewed by loading the study file on the screen. This data was automatically analysed by the software.

The displayed parameter included the RHI (reactive hyperemic index), LnRHI (Natural log of Reactive hyperemic index).

How EndoPAT measures endothelial dysfunction: It basically quantifies endothelial mediated changes in vascular tone. This is elicited by occluding the brachial artery, using a blood pressure cuff for 5 minutes. Surge of blood flow following the release of the cuff elicits a FMD. The dilatation is captured as RHI by EndoPAT as an increase in PAT signal amplitude.

Interpreting EndoPAT study report (Endoscore): The final analysis of this 15-minute EndoPAT assessment based on the ratio of the post-to pre-occlusion PAT amplitude of the tested arm, divided by the post – to pre- occlusion ratio of the control arm is refer to as an Endoscore. The higher the score the better the endothelial health and the lower the risk of heart disease. There are three categories of scores. The first category is the red zone (score of 1.68 and below). This indicate very poor endothelial function that requires immediate evaluation and need for intervention. The second category is the yellow zone (score between 1.69 and 2). This signifies that the endothelium is healthy but there is need for life style modification as the individual is not in the well -protected green zone. The third category is the green zone (score between 2.1 and 3). This signifies optimal endothelial health and protection (109).

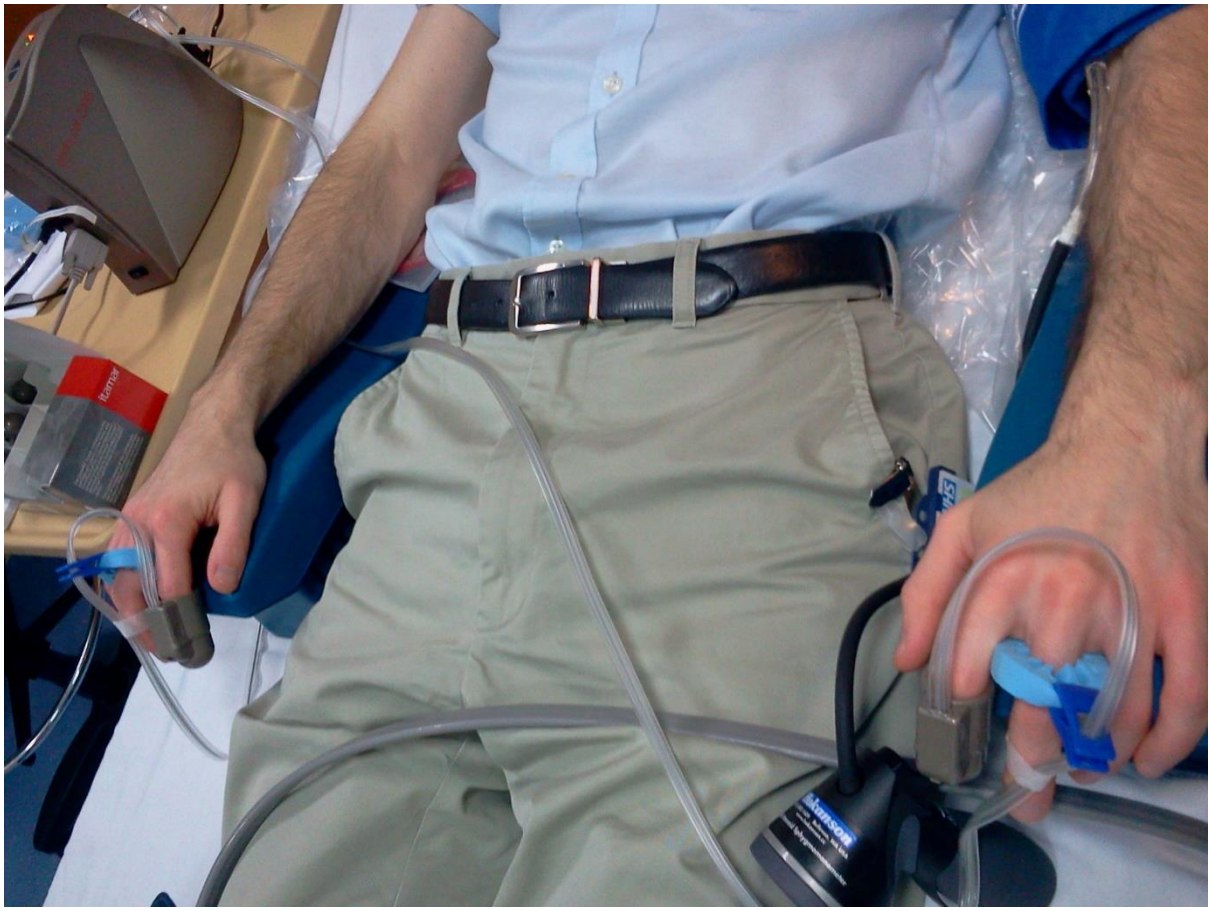


Fig. 2.1. EndoPAT machine with accessories being used on a healthy volunteer. The index fingers were held with the grey accessory clips shown above. These clips sense the wave amplitude of digital arteries (Pulse Amplitude Tonometry) and send signals to the screen. Waveforms are produced by flow mediated dilatation of the arteries following ischaemic reperfusion of the non-dependent upper limb.

2.5 Biomarker assay protocol:

Enzyme Immunoassay of 8 isoprostane

This is a form of competitive assay in which 8- isoprostane competes with an 8-isoprostane-acetylcholinesterase (AchE) conjugate for a limited number of rabbit antiserum binding sites. The enzyme immunoassay (EIA) plates (wells) were pre-coated with mouse anti rabbit IgG and the uncoated portion blocked with proprietary protein. The 8-isoprostane-AchE conjugate (tracer), 8- isoprostane standard or sample and the rabbit antiserum were added to the plate. 8 isoprostane –AchE conjugate competes with the standard or sample for binding

site on the antiserum in such a way that the more the concentration of standard or sample in the mixture, the less the number of conjugates that can bind to the antiserum site. The unbound reagent was washed off the plates and Ellman's reagent, containing AchE substrate, was then added to develop the reagent. This enzymatic reaction produces a distinct yellow colour that can be read by spectrophotometry because it absorbs strongly at 412nm. The intensity of the colour is directly proportional to the amount of 8 isoprostane-AchE conjugate bound to the well and inversely proportional to the concentration of 8 isoprostane standard or sample.

The AchE in the test kit was derived from the electric eel (Electrophorus electricus) AchE. It is a stable enzyme and capable of high turnover for hydrolysis of acetylcholine. Molecules of this AchE are covalently attached to molecules of the analyte to produce the EIA tracer (8 isoprostane- AchE conjugate). To quantify this, its reaction with Ellman reagent is measured spectrophotometrically. The Ellman reagent consist of 5, 5'-dithio-bis-(2-nitrobenzoic acid) and acetylcholine (Ach). The hydrolysis of Ach by AchE produces thiocholine which non-enzymatically reacts with 5, 5'-dithio-bis-(2-nitrobenzoic acid) to produce 5- thio-2-nitrobenzoic acid. 5- thio-2-nitrobenzoic acid has a strong absorbance at 412nm (ε=13,600). Unlike other enzymes used for enzyme immunoassay, AchE has several advantages. Firstly, it does not self-inactivate during its turnover and this allows for redevelopment of the assay if Ellman reagent spills accidentally. Secondly, AchE is stable under the assay condition with a wide pH range (5-10) and not inhibited by the buffer salt. This stable characteristic of AchE during the developmental stage means that there is no need for the use of stop solution and the plate can be read at any convenient time.

Definition of key terms

Blank: This is the background absorbance produced by Ellman reagent and this should be subtracted from all readings in each of the other wells.

Total activity: This reflects the total enzymatic activity of 8- isoprostane-AchE conjugate (tracer).

Non-Specific Binding (NSB): This reflects the non-immunologic binding of the tracer to the well. It is non-immunologic because the well does not contain antiserum.

Maximum Binding (Bo): This reflects the maximum amount of tracer that the antiserum can bind in the absence of free analyte.

Percentage Bound/Max. Bound (% B/Bo) this is the ratio of the absorbance of a standard or sample well (B) to that of the maximum binding (Bo) well.

Standard curve: This is a plot of the % B/Bo against the concentration of a series of wells containing known amount of analyte.

Dtn: This means determination and one determination is the amount of reagent used per well.

Details of enzyme immunoassay (EIA) procedure (96 well plates)

This process started with pre-assay preparation. The two main buffers used for isoprostane EIA are EIA buffer and EIA wash buffer. EIA buffer was prepared by diluting a vial of EIA concentrate (item number 40060) with 900µl of ultrapure water. The EIA wash buffer was prepared by diluting a vial of the wash concentrate (item number 40062) to 2L with ultrapure water. The ultrapure water used in this experiment is from Barnstead Nanopure Diamond machine by triplered laboratory technology, serial number SL-1254 fitted with hollow fibre

filter (lot number 75-1607A). The standardization number remained at 18.2 throughout the period of this experiment.

Samples for this experiment were collected as described above. Optimal dilution factor and interference were both checked before embarking on processing of large samples. Lack of interference was confirmed by showing that two dilutions of the samples (e.g. 1.2 versus 1.5) showed good correlation i.e. the percentage B/Bo differ by 20% or less. 8 isoprostane standard was prepared by first equilibrating the pipette tip with 100% ethanol. The same pipette tip was used to draw up 100µl of standard from a vial (item number 416354) and this was diluted to 1ml with 900 µl of ultrapure water. This new solution is referred to as bulk standard which was stable for six weeks. Eight aliquots of standard were prepared by serial dilution into tubes labelled S1 to S8. 900 µl of EIA buffer was pipetted into S1 while 750 µl was pipetted into each of tubes S2 to S8. For the serial dilution, 100 µl of solution was transferred from bulk standard to S1 and mixed thoroughly. Then, 500 µl of the solution in S1 was pipetted into S2 and mixed thoroughly. This step was repeated for tubes S3 to S8. Care was taken to ensure that the solution was thoroughly mixed after each step. These diluted standards were used the same day they were prepared. 8- Isoprostane – AchE conjugate (tracer) was prepared by reconstituting 1 vial of tracer (item number 416350) with 6ml of EIA buffer. The solution was stable for 4 weeks. 8-isoprostane EIA antiserum was prepared by reconstituting 1 vial of antiserum powder (item number 416352) with 6 ml of EIA buffer and the solution was stable for 4 weeks. Two plasma samples from two different PKD patients were used for dilution factor optimization. These samples were assayed in 4 dilutions as 1:2, 1:5, 1: 10 and 1:20. Each dilution was done in triplicate. This was followed by assay of controls and patients' samples. There was a total of 100 samples (40 controls and 60 patients). Three

96 wells plates were used for the patients and controls. Precautions taken while pipetting included making sure different tips were used for different reagents.

The pipette tips were equilibrated with the respective samples at the start of each pipetting step and pipette tips were held clear off the liquid in the wells to prevent contact. 100 µl EIA buffer was added to each of the NSB wells and 50 µl to the Bo well. 50 µl of standard from S8 was added to each of the lower standard wells. This step was repeated with the standard from S7 to S1 into the respective standard wells in duplicate. After arriving at the optimal dilution factor (1:2) the samples were prepared in 1:2 dilutions by adding 70 µl of samples to 70 µl of EIA buffer in a 1ml Eppendorf tube. 50 µl of sample was then added to each well in duplicate. 50 µl of the tracer was added to all the wells except the blank and TA wells. 50 µl of antiserum was added to all the wells except the blank, NSB and the TA wells. The wells were covered with the plastic film (item number 400012) and incubated for 18 hours at 4^0C.

Just before use, the 100dtn Ellman reagent (item number 400050) was reconstituted with 20ml of ultrapure water. The wells were emptied by lifting out the rolls from the rack one after the other. The emptied wells were washed five times with EIA wash buffer and 200 µl of Ellman reagent was added to each well. 5 µl of tracer was added to the total activity well. The plate was then covered and placed over a flat orbital shaker and allowed to develop for 2 hours at room temperature in the dark by covering it with foil material.

The bottom of the plate was wiped with clean tissue to remove any form of finger impression that might have been created while lifting it and the plate cover was gently removed to prevent the reagent spilling from the well. The surface of this cover was also checked to ensure that there are no reagent drops on it. The plate was then read using an Ascent model plate reader located in the departmental laboratory. The wavelength of this reader was set

at 405nm. The absorbance values were then printed. These values were entered in a spreadsheet provided by Cayman Chemical. The corresponding concentrations and other relevant statistics were automatically generated by this computer tool. The validity of the data was assessed by checking the regression factor of the standard curve which is expected to be as close to unity as possible. These values were entered in graphpad prism 6.

Asymmetric dimethyl arginine enzyme linked immunoassay (ADMA ELISA)

This was performed with ADMA Xpress ELISA kit from Immudiagnostik AG, Germany using plasma samples that were collected and stored as described earlier. The principle of this assay is based on competitive ELISA as described for 8 isoprostane assay. Concentration of antibody-bound tracer was inversely proportional to plasma concentration of ADMA.

All reagents and samples were prepared as instructed in the product manual, brought to room temperature (15-30 degree Celsius) and mixed well before analysis. 25 µl of standard, 25 µl of sample and 25 µl of control were added to corresponding vials. This was followed by addition of 200 µl of reaction buffer into each vial. Then, 25 µl of derivatization reagent was added into each vial and mixed thoroughly. The mixture was incubated for 30minutes on a horizontal shaker. Each of the treated sample was assayed in duplicate (50 µl of each). Prospective positions of standards, controls and samples on the 96 well plate were marked out on the protocol sheet.

Microtiter strips were prewashed five times with diluted ELISA wash buffer followed by addition of 150 µ of diluted buffer into each well. 50µl of standard, sample and control were then added into corresponding wells as marked out earlier. The plate was covered and incubated at room temperature (15-30 degree Celsius) for 2 hours on a horizontal shaker. The content of each well was then aspirated followed by thorough wash (5 times) of the wells

with diluted buffer. The plate was inverted on absorbent paper after the final wash. Then, 100 µl of diluted peroxidase conjugate was added to each well. The plate was covered and incubated for 30minutes at room temperature on a horizontal shaker. The content of each well was aspirated and washed again with diluted wash buffer and inverted on absorbent paper. This was followed by addition of 100µl of tetramethylbenzine (TMB) substrate, a peroxidase substrate to each well. The plate was then incubated for 12 minutes at room temperature in the dark. The colour change was observed intermittently for good differentiation. Finally, the reaction was stops by adding 100 µl of stop solution into each well followed by thorough mix. Absorption was determined immediately with ELISA plate reader at 450nm.

Prostaglandin 2 enzyme linked immunoassay (PGE2 ELISA)

This was performed using PGE2 assay kit from R&D systems, United Kingdom (catalog number KGE004B). The principle of this assay procedure is based on competitive ELISA. PGE2 in the sample competed with horseradish peroxidase labelled PGE2 for binding sites on a mouse monoclonal antibody. Therefore, colour change intensity was inversely proportional to concentration of PGE2 in samples. Plasma and urine (Cell free urine) samples were used for this assay.

All reagents, standards and samples were prepared as instructed in the product manual. Corresponding wells on the plate were entered on the protocol sheet for standards, control and samples. 200µL of calibrator diluent was added to the NSB wells while 150µL was added to Bo wells. 150µL of standard, control and samples were added to remaining wells. Then, 50 µL of primary antibody solution was added to all wells except NSB wells. This produced blue colouration in all, except NSB wells. Plate was sealed and incubated for 1 hour at room

temperature on a horizontal orbital microplate shaker. This was followed by addition of 50µL of PGE2 conjugate to each well, unwashed. The plate was sealed again and incubated for 2 hours at room temperature on a shaker. Then, content of each well was aspirated followed by a thorough wash (four times) and plate inversion on a clean absorbent paper. 200µL of substrate solution was added to each well followed by incubation at room temperature for 30 minutes on benchtop while protecting the content from light. Lastly, 100µL of stop solution was added to each well. Optical density was determined within 30 minutes using a plate reader (Multiskan Ascent V1.24) at 450nm wavelength.

Total nitric oxide and nitrate/nitrite colorimetric assay

This was performed using assay kit from R&D Systems (catalog number KGE001) with 2 assay options. The first option (nitrite assay) measured endogenous nitrite while the second (nitrate reduction assay) measured total nitrite. Endogenous nitrite was subtracted from total nitrite value to obtain nitrate concentration.

Firstly, serum and plasma samples were microfiltered with 10.000MW cut-off filters (Millipore, catalog number UFC501096) for deproteinization and diluted in 20-fold and 2-fold respectively. 0.5ml of high purity water was filtered through the microfilter membrane prior to deproteinization. Reagents, standards and samples were prepared as instructed in manufacturer's protocol.

Nitrite assay: 50µL of reaction diluent was added to the blank wells while the same volume of nitrite standard or sample was added to the remaining wells. This was followed by addition of 50µL of reaction diluent to all wells. Then, 50µL of Griess reagent 1 followed by another 50µL of Griess reagent ii was added to all wells. The content of each well was mixed by gently

tapping the side of the plate followed by incubation at room temperature for 10 minutes. Lastly, the optical density was determine using a microplate reader at 540nm wavelength.

Nitrate reduction assay: 50µL of reaction diluent was added to the blank wells while the same volume of nitrate standard or sample was added to the remaining wells. Then, 25µL of lyophilized reduced β-nicotinamide adenine dinucleotide (NADH) was added to all wells. This was followed by dilution of 25µL diluted nitrate reductase to all wells. Contents of the well were mixed, covered and incubated for 30 minutes at 37 degree Celsius. Following incubation, 50µL of Griess reagent 1 followed by the same volume of Griess reagent ii was added to all wells and mixed gently. Then, incubated for 10 minutes at room temperature. Lastly, the optical density of each well was determined at a wavelength of 540nm using microplate reader (Multiskan Ascent V1.24).

Matrix metalloproteinase-2 enzyme linked immunoassay (MMP2 ELISA)

This sandwich ELISA was performed using human MMP2 ELISA kit (ab100606) from Abcam, United Kingdom. Kit was stored at – 20 degrees Celsius upon receipt. Working reagents, standards and controls were treated as instructed in manufacturer's protocol. Serum samples, standards and control (in duplicate) were equilibrated to room temperature before assay. 100µL of standard and diluted serum samples (1:2) were added into appropriate wells, covered and incubated overnight at 4 degrees Celsius. The next day, solution was discarded, wells thoroughly washed (four times) and plate inverted on absorbent paper. Then, 100µL of biotinylated MMP2 detection antibody was added to each well followed by incubation at room temperature for 1 hour on a plate shaker. The wells were emptied and washed again as in the preceding step. Then, 100µL HRP-streptavidin solution was added to each well and incubated for 45 minutes on a shaker. Again, wells were emptied and washed followed by

addition of 100µL of TMB to each of them. The solution was then incubated for 30 minutes on a shaker at room temperature. Lastly, 50µL of stop solution was added to each well and absorbance read immediately with plate reader at a wavelength of 405nm.

2.6 Creatinine Clearance: measurement and limitations

Creatinine clearance (CrCl) is the volume of plasma that is cleared of creatinine per unit time. The unit of measurement is ml/min/1.73m^2. It gives an approximate value of glomerular filtration rate (GFR).

Each participant was given a clean 5 litre container and instructed on how to collect 24-hour urine sample. They were instructed not to urinate inside the container at the start of the collection process. Subsequent voidings were collected into the container. At the end of the collection process, they were asked to urinate into the container and blood sample was collected for serum creatinine measurement during their study visit which was timed to coincide with the end of the 24hour urine collection. These samples were sent to STH laboratory for measurement of CrCl.

There are limitations associated with the use of CrCl for measurement of kidney function. Firstly, it is cumbersome, and it might be difficult to ensure compliance with strict collection protocol. Spot urine collection for the estimation of albumin or protein creatinine ratio is a preferred option. Also, the procedure overestimate GFR as a result of creatinine secretion (224).

2.7 Immunohistochemistry of kidney tissue and mesenteric vessel of heterozygous Pkd2 KO mice

Immunohistochemistry is a recognised laboratory method for the detection, localization and quantification of proteins in tissues. Optimal detection of antigen is determined by antibody specificity, tissue fixation and processing. One area of difficulty is antibody specificity. For instance, using mouse primary antibody to detect antigen on mouse tissue can result in high background staining. However, there are ways of modifying the immunoreactivity of the tissue with the aim of eliminating background staining. The mouse on mouse (M.O.M) immunodetection technique utilizes a new endogenous mouse antigen blocking agent as well as a special detection method. This is one of the methods used in this thesis specifically to detect MMP2 on mouse kidney tissue as explained below. The importance of localization and quantification of biomarkers of oxidative stress is pertinent in the search for ways to halt disease progression and onset of cardiovascular events especially in a disease condition like ADPKD where cardiovascular complications have been reported as a major cause of morbidity and mortality (225). This is important because oxidative stress and endothelial dysfunction precede these cardiovascular events (226).

Immunohistochemistry was performed to investigate oxidative stress (SOD2, HO1 and 8OHdG antibodies) and endothelial dysfunction (MMP2 antibodies).

Experimental animals.

The experimental animals used for this study were $Pkd2^{+/-}$ founder mice obtained from Yale university, courtesy of Prof Somlo. They carried a null allele (ws183) for pkd2 which results from homologous recombination at exon-1 of the *Pkd2* locus (227). Details of the animal handling have been previously described (228). The left renal artery and veins of these

animals were clamped using non-traumatic clamps for 25minutes following which they were perfused for 48hours. These kidney sections were fixed with formalin and embedded in paraffin following which immunostainings were performed using protocols as described below.

Immunohistochemistry protocol for paraffin embedded sections.

Paraffin embedded tissue was prepared for staining by first dewaxing twice in xylene for 3 minutes each. This was followed by hydrating twice through 100% alcohol series for 3 min per session. The process was repeated through 95% ethanol for 3 minutes (x 1) followed by 70% ethanol for 3 minutes (x1). Then through dH2O for 3 minutes (x1). The tissue was then immersed in a solution of 3% hydrogen peroxide in methanol for 20 minutes and rinsed in distilled water (dH2O). This quenched endogenous peroxidase activity. Antigen retrieval was performed through heat induced method. The section was microwaved for 10 minutes on high power in 0.01M Tri Sodium Citrate at a pH of 6.0 and then cooled under running water. This was followed by rinsing in PBS. Then serum from Impress universal kit (Vector Systems) was used to block the section for 30 minutes followed by incubation with primary antibody for 1 hour at room temperature. (Primary at a dilution of 1/1000, made up in sterile PBS). The section was then washed with PBS thrice and incubated for 30 minutes with the secondary biotinylated antibody from Impress kit at room temperature. Washing in PBS was repeated for 5 minutes. Chromogen (DAB) was prepared as follows: 1ml of diluent + 30µl (or 2 drops) of DAB solution. The section was incubated in this chromogen preparation, observing the development under a microscope. The reaction was stopped in dH2O and counterstained for 15-30seconds with haematoxylin blue and 10 seconds in Scott's tap water. Finally, the stained

section was mounted with consul mount, protected with a cover slip and allowed to dry in room air.

Vector kits: Impress Universal Kit. DAB cat no. SK4100

Protocol for mouse on mouse immunohistochemistry

Working solutions were prepared as contained in manufacturer guide from Vector Laboratories, United Kingdom (catalogue number BMK-2202). These solutions were M.O.M-mouse IgG blocking reagent, M.O.M diluent and M.O.M biotinylated anti mouse IgG reagent.

Tissue sections were deparaffinised and hydrated through xylene, graded alcohol series and rinsed for 5 minutes as described above. Antigen unmasking was done using citrate-based solution (H-3300). Sections were incubated with 'BLOXALL' blocking solution (SP-6000) for 10 minutes to block endogenous enzyme activities. This was followed by 2 minutes' wash in PBS solution (twice). Avidin/Biotin blocking agent (SP-2001) was added to the sections. This was followed by 1-hour incubation in working solution of M.O.M mouse IgG blocking reagent and two-minute wash in PBS. Then, another 5-minute incubation in working solution of M.O.M diluent was performed. Sections were then incubated in diluted (1:1000) primary antibody (mouse monoclonal antibody) for 30 minutes after tapping off excess M.O.M diluent. This was followed by 2 minutes' wash in PBS (twice). Working solutions of M.O.M biotinylated anti mouse IgG reagent were then added to the sections and incubated for 10minutes followed by another PBS wash. Lastly, avidin based detection system was performed.

There was strict adherence to timing as described above. Also, kit was stored at 4 degree Celsius as instructed by product manufacturer.

Immunohistochemistry protocol for 8OHdG, SOD2, HO1 and MMP2.

8OHdG immunohistochemistry

Details of the methods is as described above and summarised in the table 2.1

Table 2.1: 8 OHdG immunohistochemistry

Method	IHC-paraffin embedded
Primary antibody	Goat polyclonal anti 8 OHdG (ab10802) from abcam
Negative control IgG	Goat IgG
Blocking serum	Horse serum
Secondary antibody	Horse anti goat antibody
Tissue development	DAB chromogen staining
Microscopy	Bright field, inverted microscope

SOD2 immunohistochemistry

Details of the methods is as described above and summarised in table 2.2

Table 2.2. SOD2 immunohistochemistry

Method	IHC-paraffin embedded
Primary antibody	Rabbit monoclonal antibody (D3X8F) XP, from cellsignal
Negative control IgG	Rabbit IgG
Blocking serum	Horse serum
Secondary antibody	Horse anti rabbit/mouse antibody
Tissue development	DAB chromogen staining
Microscopy	Bright field, inverted microscope

Heme oxygenase Immunohistochemistry

The details of method used for this immunostaining is as described above and summarised in table 2.3 below.

Table 2.3 Heme oxygenase immunohistochemistry

Method	IHC-paraffin embedded
Primary antibody	Rabbit polyclonal antibody (ab85309) from abcam
Negative control IgG	Rabbit IgG
Blocking serum	Horse serum
Secondary antibody	Horse anti rabbit/mouse antibody
Tissue development	DAB chromogen staining
Microscopy	Bright field, inverted microscope

Matrix metalloproteinase 2 immunohistochemistry

This is a mouse on mouse immunohistochemistry and details of the methods is described above and summarised in table 2.4 below.

Table 2.4: Matrix metalloproteinase 2 immunohistochemistry

Method	IHC-paraffin embedded, mouse on mouse
Primary antibody	Mouse monoclonal antibody (ab1818) from abcam
Negative control IgG	Mouse IgG
Blocking serum	MOM mouse IgG blocking reagent
Secondary antibody	MOM biotinylated anti-mouse antibody
Tissue development	Avidin/Biotin staining
Microscopy	Bright field, inverted microscope

2.8 **Microscopy**

Slides were viewed using the Leica DMI 4000B inverse microscope. The intensity was set to 465ms, exposure was 6.9, saturation was 44 and gain was 71. Lens power of 20 was used for microscopy. The intensity score was determined by examining 10 consecutive overlapping fields in the cortical and medullary areas of the kidney. Scoring was 1 when there was no staining and 2 when the staining was mild. Moderate staining was scored as 3 while deeply intense staining was scored as 4. Scores were entered on excel spreadsheet and the average stain intensity score calculated. These average scores were then entered in graphpad prism 6. The result was expressed as mean± SD for each slide.

CHAPTER THREE:

RESULTS.

A retrospective cross-sectional study of biomarkers of oxidative stress and endothelial dysfunction in ADPKD patients and those with other forms of chronic kidney disease compared with healthy volunteers.

3.1 Introduction

In this chapter I sought to test the hypothesis that biomarkers of endothelial dysfunction are elevated in ADPKD.

3.2 Methods

This is a single centre, cross sectional study of retrospective samples collected during previous studies by Ong et al (unpublished) The study design was described in chapter two. I acknowledge the contributions of Professor Albert Ong to this chapter. The study population consisted of sixty ADPKD patients, forty OCKD patients and thirty-six healthy volunteers.

3.3 Results.

3.3.1 Demographic characteristics of the study population

Table 3.1 shows the demographic characteristics of the study population and some clinical correlates. Among the matched healthy volunteers, 16.7% were hypertensive.

Table 3.1. Basic demographic and some clinical correlates of healthy volunteers (HV), ADPKD patients and those with other forms of chronic kidney diseases (OCKD).

Variable	**HV**	**OCKD**	**ADPKD**	**P value**
Age(y), Mean±SD	31.47±10.45	55.49±12.33	50.15±12.64	<0.001
Sex, Females, (%)	17(47.2)	15(36.6)	35(57.4)	Ns
Hypertensives, (%)	16.7	57.3	36.1	Ns
Serum Cholesterol (mmol/L)	4.71±0.99	4.28±0.95	4.64±0.78	Ns
Plasma Glucose (mmol/L)	4.48±0.65	6.30±1.93	6.23±3.41	<0.05
eGFR(ml/min/1/73m^2)	97.48±16.53	48.58±22.55	54.72±28.85	<0.001
Serum Urate (µmol/L)	309.07±85.51	409.24±119.18	376.12±108.69	0.001
Serum creatinine (µmol/L)	72.67±11.30	139.02±51.67	130.45±63.41	<0.001
Total	36	40	60	

3.3.2 8 isoprostane level in ADPKD compared with healthy volunteers or other chronic kidney disease

Plasma concentration of 8 isoprostane was assayed in patients and controls as a biomarker of oxidative stress. The mean concentration in HV was 11.4 ±10.0 pg/ml. This was significantly higher in both OCKD (18.4 ± 15.9 pg/ml) and ADPKD patients (22.6 ± 19.0 pg/ml), $p<0.05$. However, there was no statistically significant difference between OCKD and ADPKD groups, $p>0.05$ (Figure 3.1).

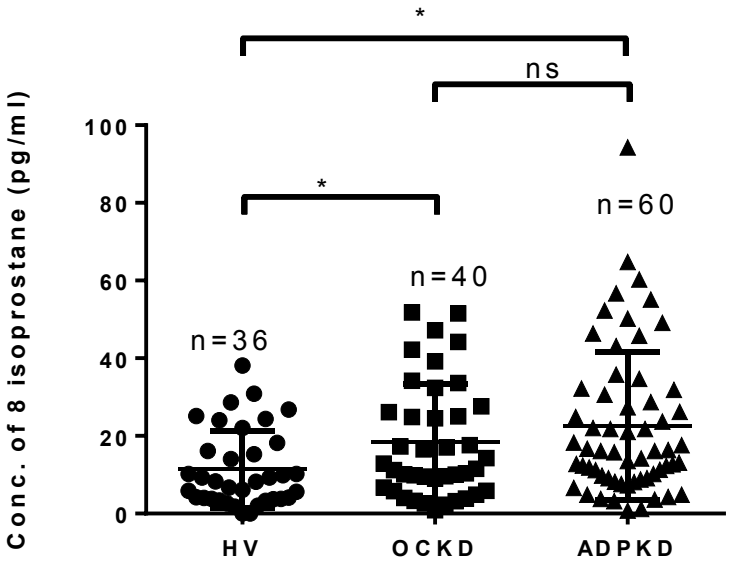


Figure 3.1. Plasma concentration of 8 isoprostane in healthy volunteers (HV), ADPKD patients and those with other forms of chronic kidney disease. (OCKD).*n=p>0.05,* =p<0.05.*

3.3.3 Asymmetric dimethyl arginine concentration in healthy volunteers, ADPKD and patients with other forms of chronic kidney disease.

The plasma level of ADMA was assayed and the concentration was presented in µmol/L in the three groups of participants. The mean plasma concentration in HV was 0.27 ± 0.15. The mean concentration in OCKD was higher at 0.37 ± 0.21. ADPKD patients had the highest mean plasma concentration which was 0.44 ± 0.39 (figure 3.2). The difference in mean concentration among the three groups of participants was statistically significant (ANOVA, $p<0.05$). Comparison of the relationship between the mean concentrations of the groups using a post hoc test (Bonferroni correction) showed that there was no statistically significant difference between the concentrations in OCKD and ADPKD patients ($p>0.05$). On the other hand, there was statistically significant difference in mean concentrations between OCKD and HV ($P<0.05$) as well as between ADPKD patients and HV ($p<0.05$).

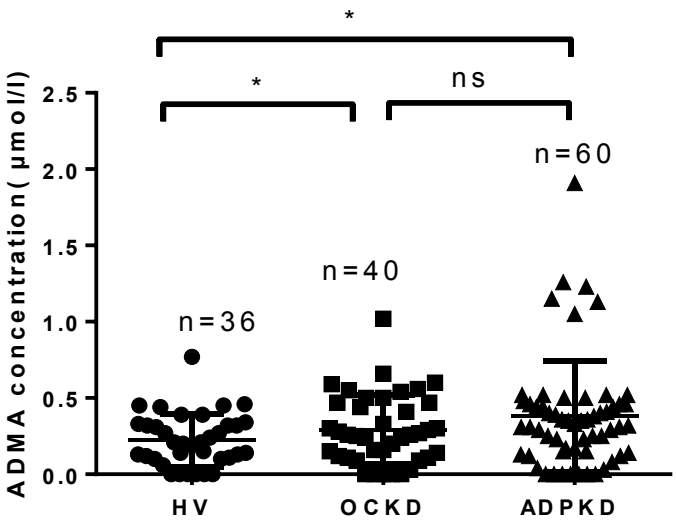


Figure 3.2:Plasma ADMA concentration in HV, ADPKD and those with other forms of chronic kidney disease*(*=p<0.05,ns=p>0.05).*

3.3.4 Correlations between 8 isoprostane and different variables in study population.

An unadjusted correlation analysis (bivariate) between 8 isoprostane and different variables in the study population was performed (Table 3.2). Systolic blood pressure correlated with plasma concentration of 8 isoprostane in ADPKD patients ($p<0.001$). There was no significant correlation between 8 isoprostane and the other variables in any of the 3 groups of participants ($p>0.05$). 8 isoprostane correlated with blood pressure but not with age, gender and kidney function.

Table 3.2 Correlation between 8 Isoprostane and different variables in HV, OCKD and ADPKD patients*. (*=p<0.05, p value greater than 0.05 was considered not statistically significant).*

Variable	Group	Pearson Correlation	P value
Age (years)	ALL	0.127	0.218
	HV	0.326	0.149
	ADPKD	0.093	0.531
	OCKD	-0.157	0.433
Sex	ALL	0.101	0.329
	HV	0.211	0.358
	ADPKD	0.180	0.226
	OCKD	-0.201	0.314
Systolic Blood Pressure	ALL	0.321	0.002*
	HV	0.505	0.144
	ADPKD	0.389	0.008*
	OCKD	0.115	0.566
eGFR	ALL	-0.102	0.330
	HV	0.241	0.307
	ADPKD	-0.096	0.522
	OCKD	0.105	0.601

3.3.5 Correlations (multivariate) between 8 isoprostane and kidney function in study population

An adjusted relationship (correcting for age of participants in years) between 8 isoprostane and eGFR in the study population was performed. There was no statistically significant relationship between 8 isoprostane and eGFR in any of the three study groups even after correcting for age of participants ($p>0.05$).

Table 3.3 Multivariate analysis of the relationship between 8 isoprostane and eGFR after correcting for age in HV, ADPKD and patients with other forms of chronic kidney disease**.** *(p value greater than 0.05 was considered not statistically significant).*

Variables	**Pearson Correlation**	**95% Confidence interval**		**P value**
		Lower Limit	Upper Limit	
ALL *Age(years)*	0.100	-0.241	0.441	0.563
eGFR(ml/min)	-0.057	-0.172	0.058	0.330
ADPKD *Age(years)*	0.021	-0.588	0.629	0.946
eGFR(ml/min)	-0.064	-0.264	0.136	0.522
OCKD *Age(years)*	-0.189	-0.854	0.476	0.563
eGFR(ml/min)	0.082	-0.237	0.402	0.601
HV *Age(years)*	0.513	-0.004	1.029	0.051
eGFR(ml/min)	0.142	-0.142	0.427	0.307

3.3.6 Correlations between asymmetric dimethyl arginine and different variables in the study population

An unadjusted relationship between ADMA and different variables was performed not just in the 3 groups of participants separately but also in all the participants combined. Bivariate correlation analysis (Pearson Correlation) showed that ADMA weakly correlate with age in all the participants combined ($R = 0.191$, $p<0.05$). However, this correlation was not significant when the relationship was analysed in each of the three groups. ($p>0.05$). Furthermore, there was significant negative correlation between ADMA and eGFR in all the study groups combined ($R = -0.261$, $p<0.01$) and in ADPKD patients ($R=-0.294$, $p<0.05$). There was no significant correlation between ADMA and eGFR in OCKD patients. As expected, there was no correlation between ADMA and serum creatinine (Table 3.4).

Table 3.4. Correlation between asymmetric dimethyl arginine (ADMA) and different variables in HV, OCKD and ADPKD patients. *(*=p<0.05 , p value greater than 0.05 was considered not statistically significant).*

Variable	Group	Pearson Correlation	P value
Age (years)	ALL	0.191	0.049*
	HV	-0.066	0.728
	ADPKD	-0.101	0.524
	OCKD	0.247	0.153
Sex	ALL	-0.105	0.287
	HV	0.031	0.871
	ADPKD	-0.217	0.173
	OCKD	-0.100	0.574
Systolic Blood Pressure	ALL	0.138	0.160
	HV	0.098	0.613
	ADPKD	0.165	0.303
	OCKD	-0.050	0.774
eGFR	ALL	-0.261	0.008*
	HV	0.115	0.284
	ADPKD	-0.294	0.031*
	OCKD	-0.166	0.174

3.3.6.1 ADMA, kidney function and gender in ADPKD patients.

Two by two contingency table was drawn from cross tabulation analysis of the relationship between ADMA and gender (Table 3.5) as well as ADMA and eGFR. (Table 3.6). To achieve this, eGFR was categorised into two (< 60ml/min/1.73m^2 and ≥60ml/min/1.73m^2). Plasma concentration of ADMA was also arbitrarily categorised into two (<0.42 µmol/L and ≥0.42 µmol/L). The basis chosen for ADMA level was the observed upper limit of plasma concentration in the healthy volunteer which was 0.42 µmol/L.

Interactions of these categorical variable was performed with chi- square test and the odds ratio was also assessed. Male ADPKD patients had about 20 percent lower risk of plasma ADMA < 0.42 µmol/L (table 3.5) but the interaction was not significant (OR = 0.82 (95% CI =0.40- 1.71, p>0.05). Also, the ADPKD patients with eGFR ≥ 60ml/min/1.73m^2 had 40% risk of their plasma ADMA being < 0.42 µmol/L when compared with those with eGFR < 60ml/min/1.73m^2 (table 3.6) but the interaction was not statistically significant (OR = 1.4 (95% CI =0.55- 3.80, p>0.05).

Table 3.5 ADMA and gender in ADPKD patients.

For ADMA 0.42µmol/L and above in males, OR = 0.82 (95% CI =0.40- 1.71, p>0.05).

ADMA (µmol/L)	**Gender**	
	Male (%)	Female (%)
<0.42	38.5	61.5
0.42 and above	46.7	53.3

Table 3.6 ADMA and eGFR in healthy volunteers, ADPKD patients with early disease**.**

For eGFR 60 and above, ADMA <0.42µmol/L, OR = 1.4 (95% CI =0.55- 3.80, p>0.05).

ADMA (µmol/L)	**eGFR(ml/min/1.73m^2)**	
	<60	**60 and above**
<0.42	**61.5%**	**38.5%**
0.42 and above	**73.3%**	**26.7%**
Total	**65.9%**	**34.1%**

3.3.7 Correlations between asymmetric dimethyl arginine and age in study population

An adjusted (correcting for age) multivariate analysis of the relationship between ADMA and age was performed (table 3.7). The correlation coefficient (R), 95% confidence interval and the p value were evaluated in all the 3 groups combined and individually. There was no statistically significant relationship between ADMA and age in any of these groups (p>0.05).

Table 3.7 Multivariate analysis of the relationship between ADMA and eGFR after correcting for age in HV, ADPKD and patients with other forms of chronic kidney disease.

Variables	**Pearson Correlation**	**95% Confidence interval**		**P value**
		Lower limit	Upper limit	
ALL *Age (years)*	0.000	-0.005	0.005	0.962
eGFR(ml/min)	-0.003	-0.005	0.000	0.057
ADPKD *Age (years)*	0.002	-0.007	0.011	0.632
eGFR(ml/min)	0.000	-0.004	0.004	0.876
OCKD *Age (years)*	0.004	-0.003	0.011	0.258
eGFR(ml/min)	0.000	-0.004	0.003	0.802
HV *Age (years)*	0.006	-0.007	0.007	0.098
eGFR(ml/min)	0.117	-0.003	0.005	0.592

3.3.8 Asymmetric dimethyl arginine, 8 isoprostane and uric acid.

The relationship between ADMA and uric acid as well as 8 Isoprostane and uric acid was performed. Pearson correlation coefficient (2 tailed assessment) was determined for each group of participants. There was no statistically significant relationship between serum uric acid and ADMA ($p>0.05$). There was also no significant correlation between serum uric acid and 8 isoprostane. ($p>0.05$) in any of the groups (table 3.8).

Table 3.8: Correlation between serum uric acid and ADMA as well as 8 isoprostane in healthy volunteers, ADPKD patients and those with other forms of chronic kidney disease. *(p value >0.05 was considered not statistically significant)*.

Group	**Pearson Correlation**	**P value**
With ADMA		
ALL	0.124	0.206
HV	0.090	0.677
OCKD	0.080	0.647
ADPKD	0.077	0.610
With 8 Isoprostane		
ALL	-0.093	0.380
HV	-0.339	0.184
OCKD	-0.011	0.958
ADPKD	-0.192	0.197

3.3.9 Kidney function and serum uric acid concentration in heathy volunteers, ADPKD patients and those with other forms of chronic kidney disease.

The relationship between kidney function (eGFR) and uric acid in the study population was assessed by performing a Pearson correlation coefficient (2 tailed). There was a significant correlation between eGFR and uric acid in OCKD patients ($p<0.05$). There was a stronger negative correlation between these two variables in ADPKD patients ($p<0.001$) and in all the participants combined ($P<0.001$).

Table 3.9 Correlation between serum uric acid and eGFR in healthy volunteers, ADPKD patients and those with other forms of chronic kidney disease. *(*=p was significant at <0.05, **= p was significant at <0.001).*

Group	Pearson correlation	P value
ALL	-0.513	<0.001**
HV	0.301	0.135
OCKD	-0.401	<0.01*
ADPKD	-0.598	<0.001**

3.3.10 Stages of chronic kidney disease in participants

Study participants were categorised into chronic kidney disease stages 1 to 5. Expectedly, all the participants under the healthy volunteer group had eGFR greater than 60ml/min/1.73m^2. (Stages 1 and 2). However, 25% (10 patients) and 36.6% (22 patients) of OCKD and ADPKD patients respectively were in CKD stages 1 and 2. I next tested the hypothesis that biomarkers of oxidative stress (8 isoprostane) and endothelial dysfunction (ADMA) were increased in

ADPKD and other forms of chronic kidney disease with early disease (eGFR≥ 60ml/min/1.73m^2).

Table 3.10 Frequency table of the percentages of participants at various stages of chronic kidney disease in the retrospective study.

Stages of CKD (ml/min)	Healthy volunteers	OCKD patients	ADPKD patients
90 and above, % (n)	67.7 (21)	10 (4)	13.3 (8)
60 to 89, % (n)	23.3 (10)	15 (6)	23.3 (14)
30 to 59, % (n)	-	47.5 (19)	41.7 (25)
15 to 29, % (n)	-	27.5 (11)	20.0 (12)
Less than 15, % (n)	-	-	1.7 (1)

3.3.11 Demographic profile and some clinical correlates for participants with preserved kidney function

Sub analysis of the demographic characteristics and clinical correlate of the study participants in CKD stages 1 and 2 was performed. The mean age± SD for the OCKD and ADPKD patients with early disease were 45.80± 12.03 years and 42. 50 ± 10.16 years respectively while that of healthy volunteers in the same stages of kidney disease was 31.19± 10.21years. There was significant difference between the mean age of participants ($p<0.001$) and the mean eGFR ($p<0.05$). Conversely, there was no significant difference in gender among the study group.

Mean plasma glucose was 4.48 ± 0.85 mmol/L. It was higher in ADPKD and OCKD patients with preserved kidney function and there was statistically significant difference in the mean plasma glucose (one -way ANOVA) among the three groups ($p<0.05$).

3.3.12 Plasma 8 isoprostane in participants with preserved kidney function.

A sensitive analysis of data that excludes OCKD and ADPKD patients with eGFR greater than 60ml/min/1.73m^2 was performed (figure 3.3) There was no statistically significant difference in the mean plasma level of 8 isoprostane among the three groups of participants (ANOVA, $p>0.05$). Post hoc analysis (Bonferroni procedure) showed that there was no significant difference in mean concentration 8 isoprostane between ADPKD patients and healthy volunteers as well as between OCKD patients and healthy volunteers. ($p>0.05$).

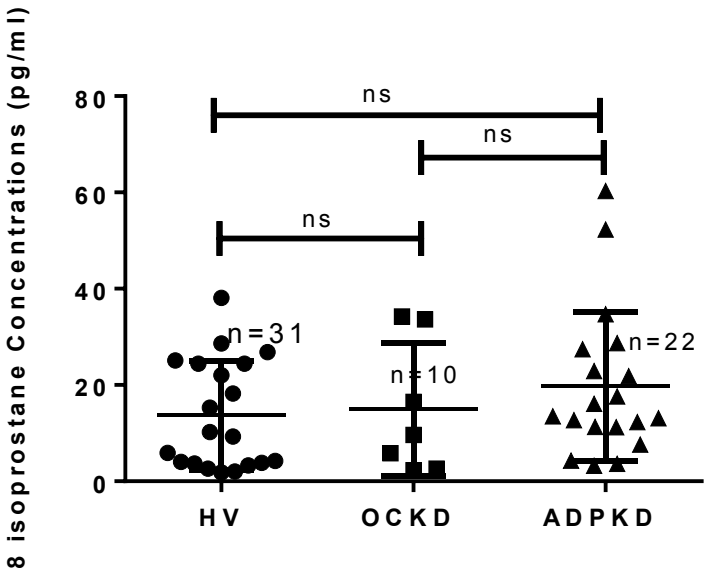


Figure 3.3: Plasma concentration of 8 isoprostane in ADPKD and OCKD patients with early disease and healthy volunteers (ns=p>0.05).

3.3.13 Plasma ADMA in participants with preserved kidney function.

The mean plasma levels of ADMA in the three groups of participants with eGFR greater than 60ml/min/1.73m^2 were as shown in figure 3.4. There was no statistically significant difference among the three groups (One-way ANOVA, $p>0.05$). Post hoc analysis showed that there was no significant difference between ADPKD patients and healthy volunteers ($p<0.05$) as well as between OCKD patients and healthy volunteers ($p<0.05$).

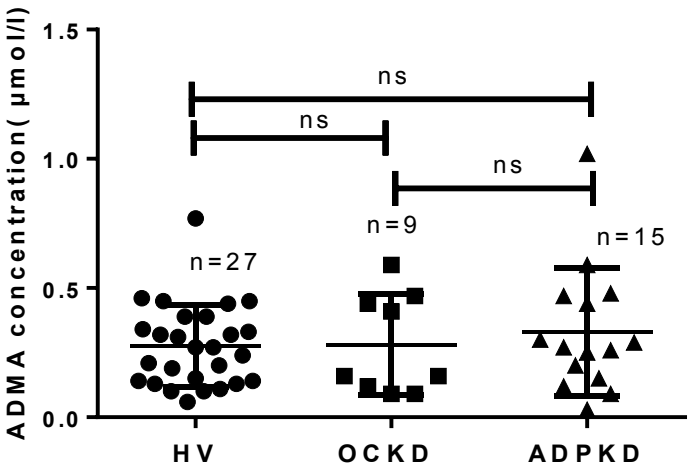


Figure 3.4. Plasma concentration of ADMA in ADPKD, OCKD patients with early disease and healthy volunteers (ns=p>0.05)

3.4 DISCUSSION.

In this chapter, one of my major findings was that oxidative stress as measured by plasma level of epi-$PGF_{2\alpha}$ (8 isoprostane) was greater in ADPKD patients than healthy controls. Also, patients with other forms of chronic kidney disease also had significantly higher level of 8 isoprostane when compared with healthy volunteers (figure 3.1). I found no significant difference in the concentration of 8 isoprostane between ADPKD and non-ADPKD chronic kidney disease patients. This was like reports in earlier studies of oxidative stress in ADPKD patients. Klawitter and colleagues reported a significant increase in the serum level of 8 isoprostane in ADPKD patient cohort from the HALT study (199). Similarly, Menon and colleagues reported an increase in level of 8 isoprostane in ADPKD patients with early disease from a study of inflammation, oxidative stress and insulin resistance (170). I next analysed the data by stages of chronic kidney disease and did not find any significant difference in the level of this biomarker in ADPKD patients with early disease compared with healthy volunteers. Another major finding was that endothelial dysfunction as measured by plasma level of ADMA was significantly higher in ADPKD patients than healthy controls but there was no difference in the levels when compared with patients with non ADPKD chronic kidney disease. Furthermore, serum Uric acid correlated with kidney function but not with plasma ADMA nor epi-$PGF_{2\alpha}$ (8 isoprostane) and plasma ADMA correlated with level of kidney function as measured by eGFR. In those with early disease, I found no statistically significant difference in oxidative stress as measured by plasma level of epi-$PGF_{2\alpha}$ (8 isoprostane) among the 3 groups of participants with eGFR≥60ml/min/1.73m^2 and there was also no statistically significant difference in endothelial dysfunction as measured by plasma level of asymmetric dimethyl arginine among the 3 groups.

Endothelial dysfunction has been reported to precede cardiovascular events in other disease conditions as well as in patients with chronic kidney disease. In this study of ADPKD and non-ADPKD CKD patients, oxidative stress as well as endothelial dysfunction were found to be present when compared with healthy volunteers. Conversely, when analysis of data was performed such that results from only patients with early stage diseases were considered, patients with ADPKD and non-ADPKD CKD were found to have no endothelial dysfunction and no significant oxidative stress as compared with healthy volunteers. This is at variance with majority of reports in the literature.

However, asymmetric dimethyl arginine and serum uric acid were found to correlate with kidney function in our studied population. This is in keeping with findings in the literature(212).

In addition, blood glucose concentration has been reported to be significantly higher in patients with ADPKD (229) as well as in non ADPKD CKD patients (230). These agree with my finding of significantly higher blood glucose concentration in these group of patients.

CHAPTER FOUR

Investigation into endothelial dysfunction in early stage disease (CKD 1 and 2) ADPKD patients and age, sex and eGFR matched healthy volunteers.

4.1 Introduction.

The hypothesis in this chapter is that endothelial dysfunction exists in normotensive early disease (eGFR≥60ml/min) ADPKD patients. In the previous chapter, ADPKD patients group comprised of early and late disease participants. Also, confounding factors like hypertension were also not excluded.

The aim was to investigate endothelial dysfunction in ADPKD with the objective of finding out if the EndoPAT machine could detect ED and if there is elevation in concentration of biomarkers of ED and oxidative stress in early disease patients without confounding factors. I acknowledge the interest and time of all ADPKD patients and healthy volunteers who responded to my invitation and participated in this prospective study.

4.2 Methods

This was a single centre prospective study involving patients attending a dedicated outpatient clinic at the Sheffield Kidney Institute. I investigated ED using the non-invasive method known as pulse arterial tonometry (ENDOPAT 2000). I also assayed biomarkers of ED (ADMA and nitrite/nitrate) as well as biomarkers of oxidative stress (8 isoprostane and PGE2).

Details of the method was as described earlier in chapter 2.

4.3 Results

4.3.1 Demographic characteristics and clinical correlates of participants.

Basic demographic characteristics of the study participants are shown in table 4.1. There was no statistically significant difference between the mean age and gender of healthy volunteers and the ADPKD patients.

Table 4.1. Basic demographics and some clinical correlates in normotensive ADPKD patients with early stage disease and healthy volunteers. (ns=p>0.05).

Variables	**HV**	**ADPKD**	**P value**
Age(y), Mean±SD	29.65±9.0	33.78±8.14	ns
Sex, Female (%)	10 (50)	11(61.1)	ns
Body mass index (kg/m^2)	25.1±2.91	24.71±4.05	ns
Serum Cholesterol (mmol/L)	4.68±0.77	4.83±0.77	ns
Serum urate (µmol/L)	294.53±81.83	294.17±83.50	ns
Phosphate (mmol/L)	1.06±0.15	1.06±0.12	ns
Serum calcium (mmol/L)	2.27±0.05	2.28±0.06	ns
Calcium phosphate product ($mmol^2/L^2$)	2.42±0.34	2.42±0.29	ns
Serum creatinine (µmol/L)	68.87±13.92	74.22±12.11	ns
C -Reactive Protein (mg/dl)	1.9±2.6	1.8±2.0	ns
Total	20	18	ns

4.3.2 Some cardiovascular risk factors in normotensive ADPKD patients with early disease and healthy volunteers

The mean 24hour urine protein, plasma homocysteine, diastolic blood pressure and creatinine clearance in the heathy volunteers and ADPKD patients are shown in figures 4.2 to 4.4.

Blood pressure in normotensive ADPKD patients with early stage disease and healthy volunteers.

Figure 4.2 shows the diastolic blood pressure (DBP) in normotensive ADPKD patients and healthy volunteers. The mean DBP in patients was 78.00 ± 8.94 mmHg. This was higher than the mean value in healthy volunteers, 68.15 ± 6.57 mmHg and this difference was statistically significant (p< 0.001).

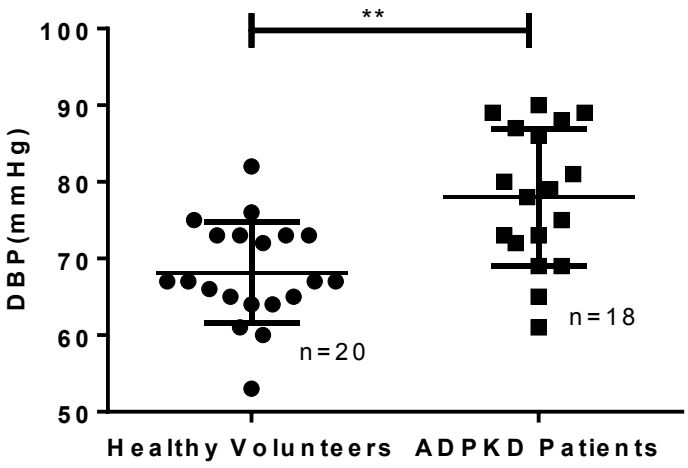


Figure 4.1: Diastolic blood pressure in normotensive ADPKD patients and healthy volunteers (**:p<0.001, DBP: diastolic blood pressure).

Proteinuria in normotensive early stage disease ADPKD patients and healthy volunteers.

The mean 24-hour urine protein in normotensive ADPKD patients (CKD stages 1 and 2) and healthy volunteers were as shown in figure 4.3. The concentration was significantly higher in normotensive ADPKD patients with early stage disease ($p<0.001$).

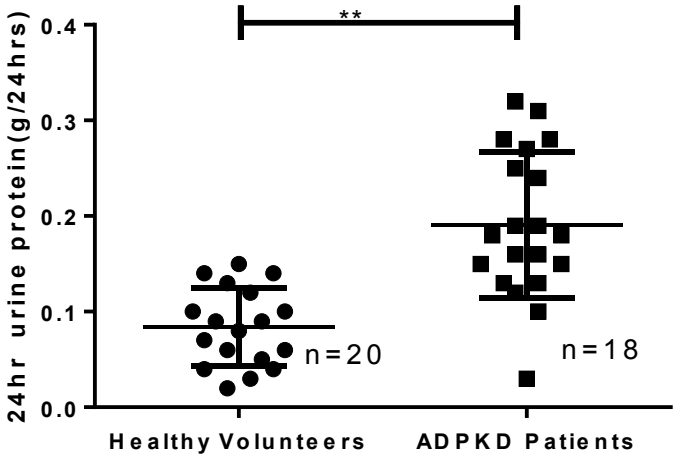


Figure 4.2: Proteinuria in normotensive ADPKD patients with early diease and healthy volunteers.(**:p<0.001)

Plasma homocysteine in early stage disease ADPKD patients with normal blood pressure and healthy volunteers

Plasma level of homocysteine was higher in normotensive ADPKD patients with early disease, 10.09 ± 3.29 mmol/L than in healthy volunteers, 9.09 ± 1.76 mmol/L but this difference was not statistically significant ($p<0.05$).

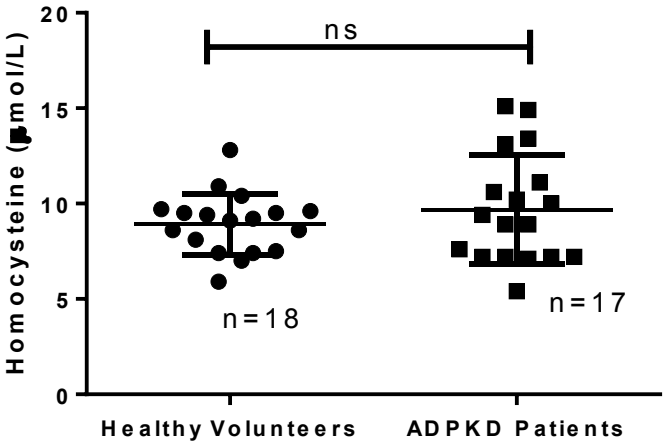


Figure 4.3: Plasma homocysteine concentration in normotensive ADPKD patients in late disease and healthy volunteers (p>0.05).

4.3.3 Reactive Hyperemia Index as a measure of endothelial dysfunction in normotensive ADPKD patients with early stage disease and healthy volunteers.

The mean RHI in the patients was 2.47±0.13 and that in the healthy control was 2.34±0.10. There was no statistically significant difference in the value between patients and healthy volunteers (p>0.05).

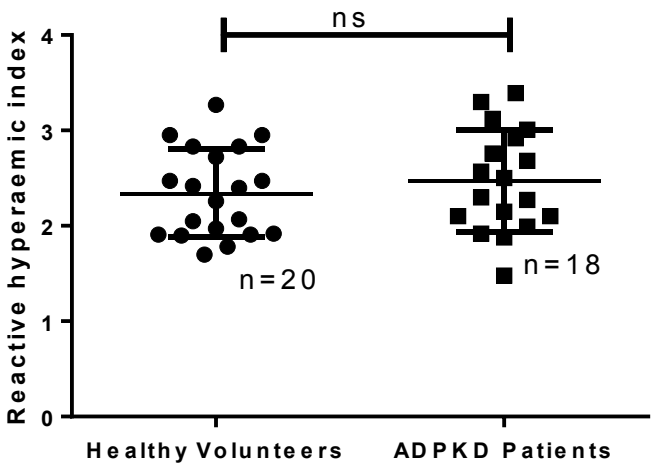


Figure 4.4: Reactive hyperaemic index in normotensive ADPKD patients with early disease and healthy volunteers (ns:p>0.05)

4.3.3.1 Correlation between RHI and some risk factors of endothelial dysfunction in normotensive ADPKD patients with early stage disease and healthy volunteers

Univariate correlation between reactive hyperemic index and some risk factors was performed. There was no significant correlation with any of the cardiovascular risk factors as shown in table 4.2. In early stage disease as well as in healthy volunteers, RHI did not correlate with any of these cardiovascular risk factors.

Table 4.2: Univariate analysis of the relationship between reactive hyperaemic index and some risk factors of endothelial dysfunction in normotensive ADPKD patients with early disease and healthy volunteers. *(p value greater than 0.05 was considered not statistically significant).*

Groups	**Pearson correlation**	**P value**
DBP		
BOTH	0.215	0.098
ADPKD	0.282	0.128
HV	0.016	0.474
Homocysteinemia		
BOTH	-0.343	0.080
ADPKD	0.424	0.097
HV	0.236	0.189
eGFR		
BOTH	-0.096	0.571
ADPKD	-0.332	0.089
HV	0.340	0.077
Proteinuria		
BOTH	0.135	0.231
ADPKD	-0.322	0.096
HV	0.115	0.341

4.3.3.2 RHI and Cardiovascular risk factors.

Multivariate correlation of RHI as the dependent variable and cholesterol, BMI, homocysteine as independent variables showed that there was no significant association between RHI and these cardiovascular risk factors in normotensive ADPKD patients. ($p>0.05$). In early stage disease as well as in healthy volunteers, RHI did not independently predict any of these cardiovascular risk factors.

Table 4.3: Multivariate analysis of the relationship between RHI as a measure of ED and some markers of cardiovascular risk in early disease ADPKD patients.

(P value greater than 0.05 was considered not statistically significant, ¶= proteinuria).

Variables	**Coefficient**	**95% CI**	**P value**
24hr urine protein¶	-0.574	-9.931 to 0.887	0.107
Homocysteine	-0.583	-0.212 to -0.024	0.097
Body mass index	0.093	-0.121 to 0.151	0.795
Cholesterol	0.276	-0.383 to 0.812	0.414

4.3.3.3 Reactive hyperaemia index and Creatinine clearance in normotensive ADPKD patients and healthy volunteers.

The mean creatinine clearance (CrCl) in patients and controls were as shown in figure 4.6. No significant association was found between creatinine clearance and eGFR between healthy volunteers and early disease ADPKD patients as shown in table 4.4. There was no significant correlation between RHI and CrCl in early disease ADPKD patient and in healthy volunteer in a bivariate Pearson correlation (table 4.5) and multivariate model (table 4.6)

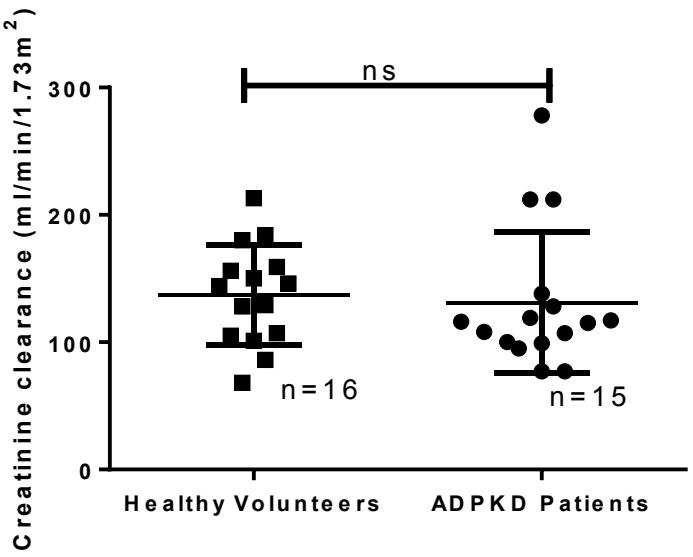


Figure 4.5: Creatinine clearance in normotensive ADPKD patients with early stage disease and healthy volunteers (ns:p>0.05).

Table 4.4 Relationship between eGFR and Creatinine clearance in ADPKD patients and healthy volunteers. *(p value greater than 0.05 was considered not statistically significant).*

Group	**Pearson correlation**	**P value**
ADPKD	0.436	0.800
Healthy volunteer	0.215	0.551

Table 4.5. Correlation between RHI and creatinine clearance.

Group	**Pearson correlation**	**P value**
BOTH	-0.266	0.074
ADPKD	-0.427	0.050
Healthy volunteers	-0.001	0.500

Table 4.6. Multivariate analysis of correlation between RHI and creatinine clearance.

Variables	**Coefficients**	**95% Confidence interval**		**P value**
BOTH	-0.118	-0.005	0.002	0.489
ADPKD	-0.242	-0.007	0.002	0.268
Healthy volunteers	0.050	-0.008	0.009	0.874

4.3.3.4 Reactive Hyperaemia Index as a measure of endothelial dysfunction in hypertensive and diabetic non ADPKD patients (Positive controls)

To confirm that the EndoPAT machine can detect ED in individuals with known cardiovascular risk factor. I measured Endoscore of 2 patients with known diagnosis of both hypertension and diabetes mellitus.

The mean RHI in the patients (n=2) was 3.45± 0.71. There was ED in these 2 patients.

4.3.4 Matrix metalloproteinase 2 enzyme linked immunoassay (MMP2 ELISA)

4.3.4.1 Serum level of MMP2.

The mean serum concentration of MMP2 in normotensive ADPKD patients was 34.58±4.39 ng/ml and this was higher than the concentration in healthy volunteers, 27.49±3.07 (Figure 4.7). However, this difference was not statistically significant ($p>0.05$).

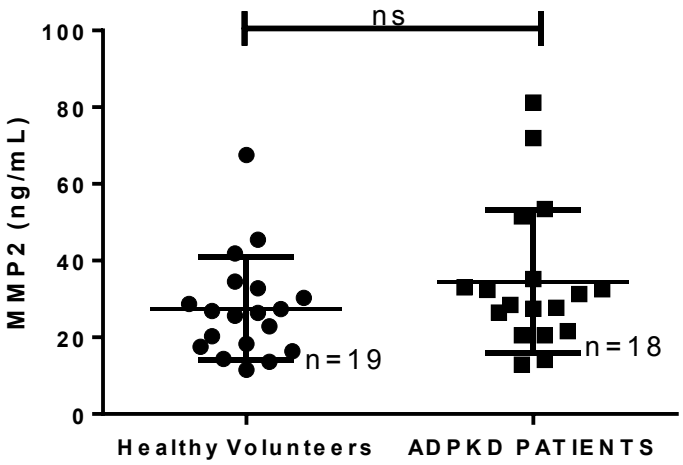


Figure 4.6: Serum matrix metaloproteinase 2 in normotensive ADPKD patients with early disease and healthy volunteers (ns:p>0.05).

4.3.4.2 MMP2 and some risk factors of endothelial dysfunction.

Pearson correlation was performed to determine the relationship between MMP2 and some risk factors of endothelial dysfunction (table 4.7). The correlation between MMP2 and diastolic blood pressure was statistically significant in the general population of participants (r=0.338, p=0.029) but not in the individual groups. (p>0.05).

Plasma homocysteine, eGFR and 24-hour urine protein had no significant correlation with serum MMP2 in the general population of participants, early disease ADPKD and healthy volunteers (p>0.05).

Table 4.7: Correlation between MMP2 and some risk factors of endothelial dysfunction in normotensive ADPKD patients with early stage disease and healthy volunteers.

*(DBP=Diastolic blood pressure, *= p<0.05, p value greater than 0.05 was considered not statistically significant).*

Groups	**Pearson correlation**	**P value**
DBP		
BOTH	0.338	0.029*
ADPKD	0.406	0.075
HV	-0.007	0.486
Homocysteinemia		
BOTH	0.111	0.604
ADPKD	0.243	0.499
HV	-0.131	0.655
eGFR		
BOTH	0.059	0.764
ADPKD	0.244	0.401
HV	-0.048	0.871
Proteinuria		
BOTH	0.203	0.300
ADPKD	-0.036	0.902
HV	-0.199	0.495

4.3.5 Asymmetric dimethyl arginine in ADPKD patients with early disease and healthy volunteers

Three variations of participants were created (figure 4.8 to 4.13)). variation 1 comprised of ADPKD patients with early stage disease, age ≤ 60year but included 2 patients whose body mass index was

greater than 30kg/m^2 (obese). Variation 2 comprised of participants in variation 1 but with the 2 obese patients excluded while Variation 3 comprised variation 2, aged ≤ 50 years.

Samples from healthy volunteers as well as the patients were either preserved with anti-oxidant, butylated hydroxytoluene (BHT) or without.

4.3.5.1: Plasma concentration of ADMA in ADPKD patients and healthy volunteers

Mean concentration of ADMA was higher in ADPKD patients when samples were not preserved with BHT for participants in variation 1 and 2 (0.66 ± 0.33 Vs 0.56 ± 0.03) and (0.65 ± 0.03 Vs 0.56 ± 0.03 μmol/L) respectively. These differences were statistically significant. ($p<0.05$) as shown in figure 4.8 and 4.9

Also, comparison of mean concentration of variation 3 with samples not preserved with antioxidant was performed (figure 4.10). There was no significant difference in plasma concentration of ADMA between early stage disease ADPKD patients, aged ≤50 years and their heathy control counterparts ($p>0.05$).

Unpaired t test analysis of participants in variation 1 (figure 4.11), 2 (figure 4.12) and 3 (figure 4.13) whose samples were preserved with antioxidants showed that there was no significant

difference between plasma concentrations of ADMA both in ADPKD patients and healthy controls ($p>0.05$).

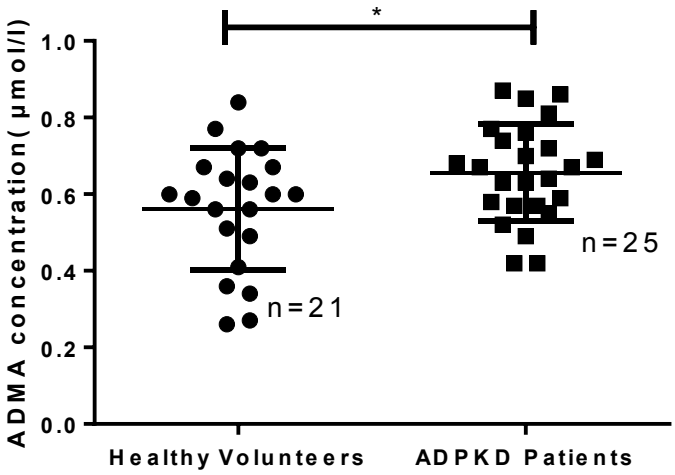


Figure 4.7: (variation 1) Plasma ADMA (NO BHT) in normotensive ADPKD patients with early and healthy volunteers (*:$p<0.05$).

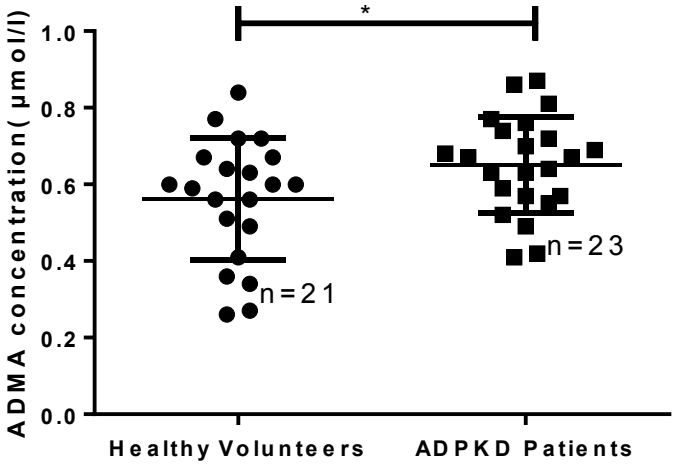


Figure 4.8:(variation 2).Plasma ADMA (NO BHT) in normotensive ADPKD patients with early disease and healthy volunteers (*:$p<0.05$).

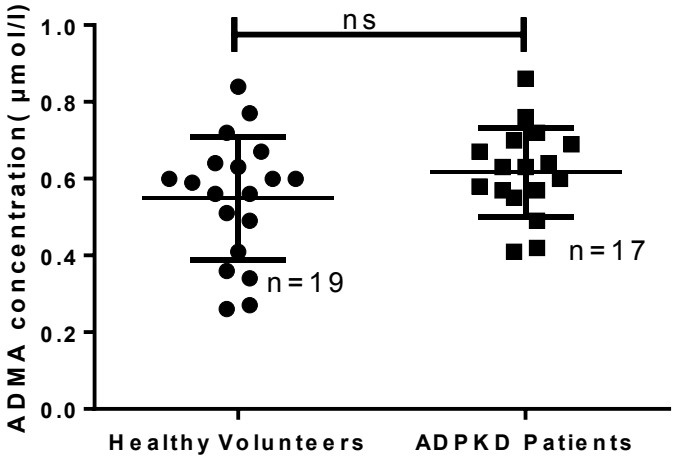


Figure 4.9: (variation 3). Plasma ADMA (NO BHT) in normotensive ADPKD patients with early disease and healthy volunteers (ns:p>0.05).

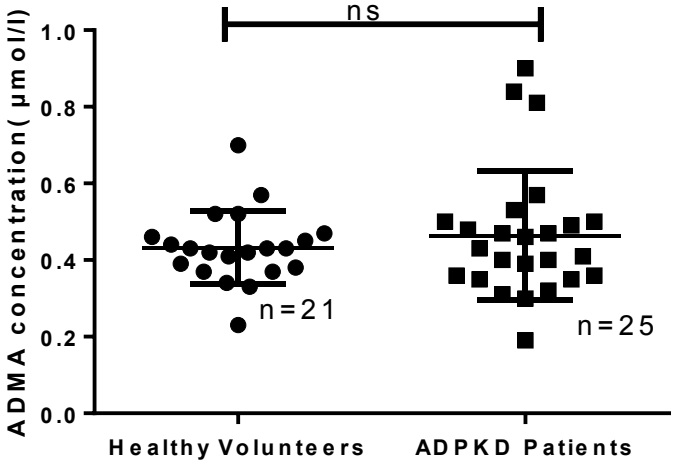


Figure 4.10 (variation 1). Plasma ADMA (WITH BHT) in normotensive ADPKD patients with early disease and healthy volunteers (ns:p>0.05).

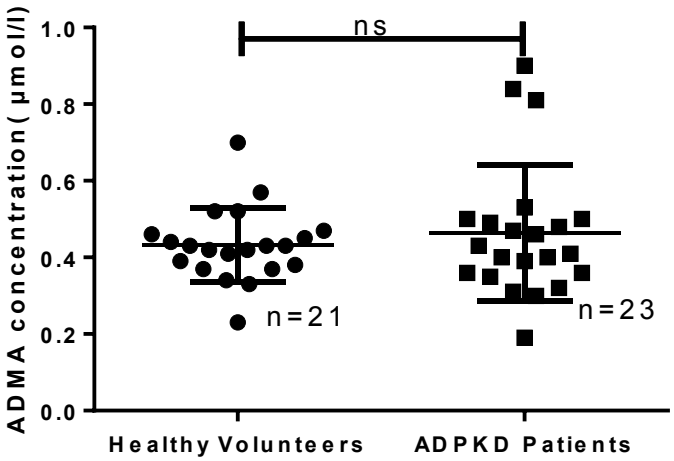


Figure 4.11: (variation 2). Plasma ADMA (WITH BHT) in normotensive ADPKD patients with early disease and healthy volunteers (ns:p>0.05).

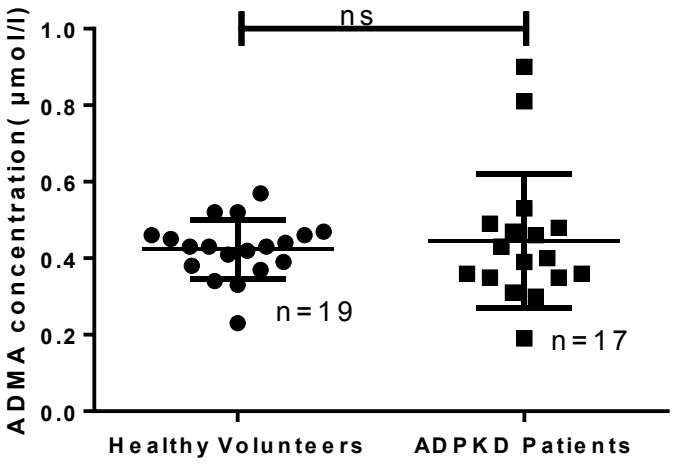


Figure 4.12: (variation 3).Plasma ADMA (with BHT) in normotensive ADPKD with early disease and healthy volunteers (ns:p>0.05).

4.3.5.2: Relationship between ADMA and some cardiovascular risk factors.

Pearson bivariate correlation was performed to determine the relationship between plasma ADMA in normotensive ADPKD with early stage disease, aged ≤ 50years and eGFR, age and sex matched healthy volunteers as well as both groups together (table 4.8). There was no statistically significant relationship between plasma ADMA and DBP, proteinuria, homocysteinemia and RHI in any of the three groups ($p>0.05$).

Table 4.8: Correlation between ADMA and risk factors of endothelial dysfunction in ADPKD and healthy volunteers. *(P value greater than 0.05 was considered not significant).*

Groups	**Pearson correlation**	**P value**
DBP		
BOTH	0.219	0.207
ADPKD	0.293	0.271
HV	0.111	0.650
Proteinuria		
BOTH	-0.074	0.712
ADPKD	-0.319	0.267
HV	0.200	0.513
Homocysteinemia		
BOTH	-0.109	0.619
ADPKD	-0.181	0.667
HV	-0.061	0.830
RHI		
BOTH	0.271	0.126
ADPKD	0.438	0.117
HV	0.017	0.145

4.3.6 8 Isoprostane as a biomarker of oxidative stress in normotensive ADPKD patients with early disease.

Mean plasma concentration of 8 isoprostane in patients and control was 13.4±2.3 and 14.07±2.1pg/ml (figure 4.14). There was no statistically significant difference between patients and controls. (p>0.05, 95% CI: -5.675 to 7.064).

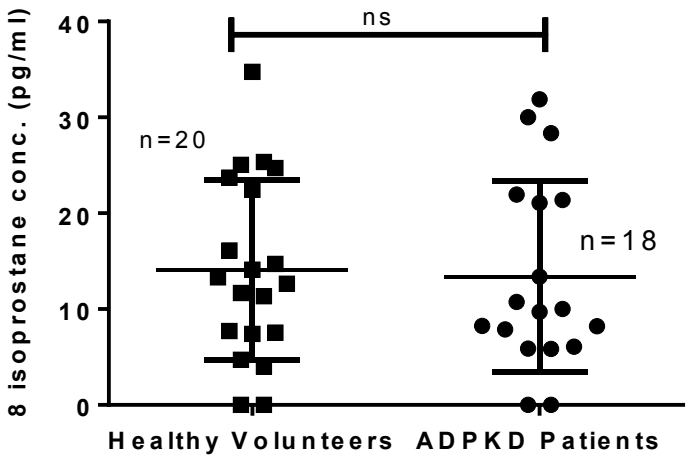


Figure 4.13: Plasma 8 isoprostane (with BHT) in normotensive ADPKD patients with early stage disease and healthy volunteers (n>0.05).

4.3.6.1: Relationship between 8 isoprostane and some cardiovascular risk factors in ADPKD patients and healthy volunteers

Correlation analysis (bivariate, Pearson) was performed to determine the relationship between plasma 8 isoprostane and DBP, proteinuria, homocysteinemia as well as RHI in both groups together and separately. There was no significant relationship between 8 isoprostane and any of these cardiovascular risk factors in any of the three groups.

Table 4.9: Correlation between 8 Isoprostane and some risk factors of endothelial dysfunction in normotensive ADPKD patients and healthy volunteers. *(P value greater than 0.05 was considered not statistically significant).*

Groups	**Pearson correlation**	**P value**
DBP		
BOTH	0.219	0.207
ADPKD	0.293	0.271
HV	0.111	0.650
Proteinuria		
BOTH	-0.074	0.712
ADPKD	-0.319	0.267
HV	-0.200	0.513
Homocysteinemia		
BOTH	-0.109	0.619
ADPKD	-0.181	0.667
HV	-0.061	0.830
RHI		
BOTH	0.271	0.126
ADPKD	0.438	0.117
HV	0.017	0.947

4.3.7. Prostaglandin 2 enzyme linked immunoassay (PGE2 ELISA)

Urine concentration of PGE2 was assayed in normotensive, ADPKD patients with early stage disease, eGFR≥60ml/min/1.73m^2 and aged ≤50year.

4.3.7.1: Urine concentration of PGE2 in ADPKD patients and healthy volunteers

Figure 4.15 shows urine concentration of PGE2 in normotensive ADPKD patients with eGFR≥60ml/min/1.73m^2 and that of age, sex and eGFR matched healthy volunteers. The concentration in ADPKD patients was not significantly different from that of healthy volunteers (1126 ±158.8 vs 1278±190.2pg/ml, p> 0.05).

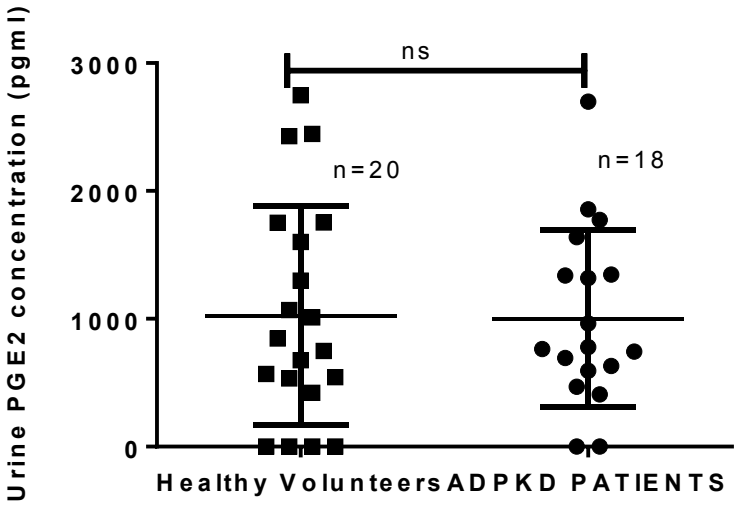


Figure 4.14: Urine concentration of PGE2 in normotensive ADPKD patients with early stage disease and healthy volunteers.(ns:p>0.05).

4.3.7.2: Correlation between urine PGE2 and some cardiovascular risk factors in ADPKD patients and healthy volunteers.

Pearson correlation was performed to assess the relationship between urine PGE2 and kidney function (eGFR) as well as reactive hyperemic index (RHI). There was no statistically significant difference between urine PGE2 and these factors (p>0.05) as shown in table 4.10.

Table 4.10 Correlation between urine PGE2 and RHI as well as eGFR in normotensive ADPKD patient with early disease and healthy volunteers.

(P>0.05 is considered not significant, HV: Healthy volunteer, ADPKD: Autosomal dominant polycystic kidney disease).

Group	**Pearson Correlation**	**P value**
RHI		
ALL	-0.117	0.529
HV	-0.249	0.351
ADPKD	0.028	0.922
eGFR		
ALL	-0.004	0.985
HV	0.164	0.593
ADPKD	-0.185	0.510

Table 4.11 shows the correlation (Pearson, bivariate) between urine PGE2 and some cardiovascular risk factors (diastolic blood pressure, homocysteinemia, body mass index and proteinuria). There was no statistically significant association between PGE2 and any of these factors (p>0.05).

Table 4.11: Correlation between Urine PGE2 and indices of cardiovascular health in normotensive ADPKD patients with early stage disease and healthy volunteers. *(P value greater than 0.05 were considered not statistically significant).*

Group	**Pearson correlation**	**P value**
DBP		
ALL	-0.042	0.821
HV	0.092	0.735
ADPKD	-0.046	0.871
Homocysteinemia		
ALL	0.063	0.769
HV	0.402	0.154
ADPKD	-0.410	0.145
Proteinuria		
ALL	-0.001	0.995
HV	0.200	-0.512
ADPKD	0.131	0.641

4.3.7.3 Plasma concentration of PGE2 in ADPKD patients with early disease and healthy volunteers.

Plasma concentrations of PGE2 in normotensive ADPKD patients with early disease (eGFR≥60ml/min/1.73m^2) and that of age, sex and eGFR matched healthy volunteers were as shown in figure 4.16. There was no significant difference between the two groups (1386±301.7 vs 1338±199.7pg/ml, p>0.05).

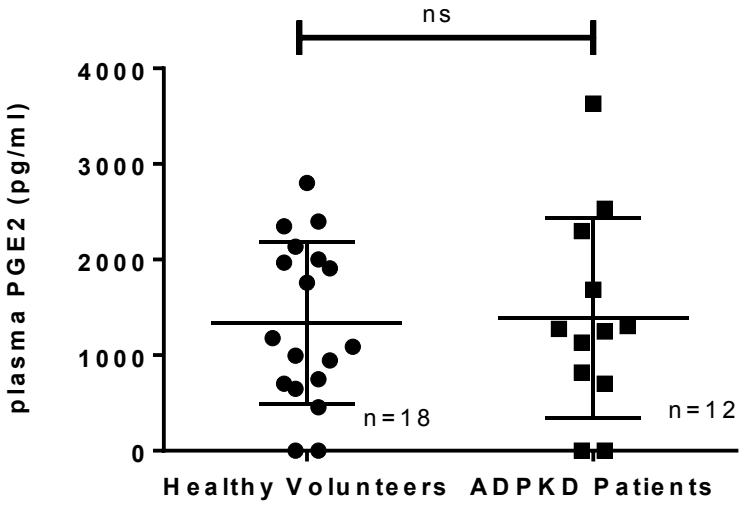


Figure 4.15: Plasma PGE2 concentrations in normotensive ADPKD patients with early disease and healthy volunteers (ns=P>0.05)

4.3.8 Total Nitric oxide and Nitrate/Nitrite

Total nitric oxide and Nitrate/nitrite concentrations were assayed in healthy volunteers and normotensive ADPKD patients with early stage disease, eGFR≥60ml/min/1.73m^2 and aged ≤50years.

4.3.8.1. Serum concentration of total nitric oxide in ADPKD patients and healthy volunteer.

Serum concentration of total nitrate/nitrite in healthy volunteers was higher than in normotensive early disease ADPKD patients and this difference was statistically significant. (44.08 ± 15.78 vs 33.54 ± 7.42 µmol/L, p< 0.05).

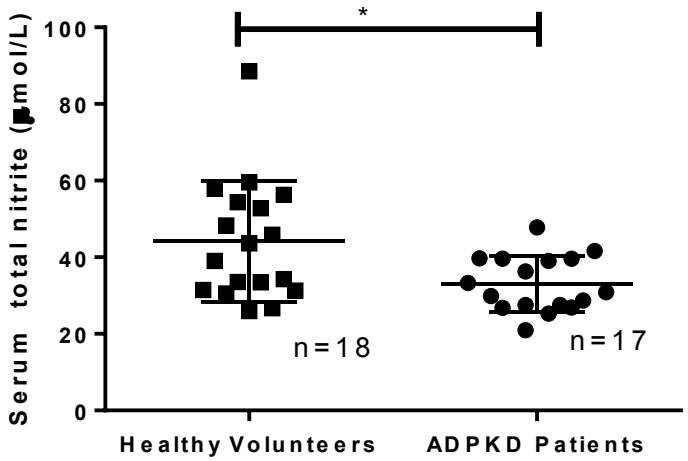
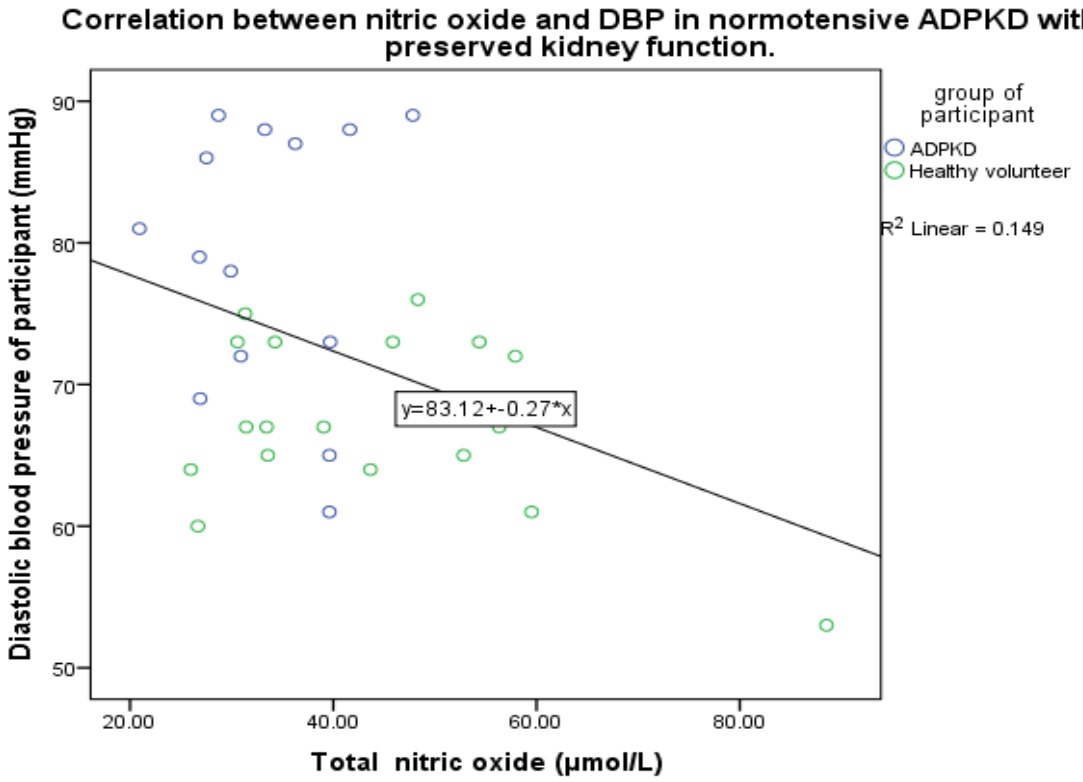


Figure 4.16. Serum total nitrite concentration in healthy volunteers and normotensive ADPKD patients with early stage disease(*:p<0.05).

4.3.8.2. Relationship between serum total nitric oxide and diastolic blood pressure in ADPKD patients and healthy volunteers.

Figure 4.18 shows the correlation between total nitric oxide and diastolic blood pressure in normotensive ADPKD with early stage disease and healthy volunteers. Total nitric oxide decreased as blood pressure increased and this relationship was statistically significant (r= -0.386, p<0.05).

Figure 4.17. Total nitric oxide and diastolic blood pressure



Correlattion between serum total nitric oxide and DBP in normotensive early disease ADPKD patients (eGFR ≥60m//min. There is significant association between the two.($p<0.05$, R=-0.386, ADPKD: Autosomal dominant polycystic kidney disease).

4.3.8.3. Relationship between serum total nitrite/nitrate and some cardiovascular risk factors in ADPKD patients and healthy volunteers.

Correlation (Pearson) between serum nitrite/nitrate concentration and some cardiovascular risk factors in normotensive ADPKD patients with early stage disease and healthy volunteers was as shown in table 4.12. No statistically significant association was observed between nitrite/nitrate and proteinuria, ADMA, RHI as well as homocysteinemia ($p>0.05$).

Table 4.12: Correlation between serum total nitrite/nitrate and some cardiovascular risk factors in normotensive ADPKD patients with early stage disease and healthy volunteers. *(P value greater than 0.05 was considered not statistically significant).*

Factors	**Groups**	**Pearson correlation**	**P value**
Proteinuria	Both	-0.152	0.449
	ADPKD	-0.105	0.721
	Healthy volunteers	0.280	0.354
ADMA	Both	0.067	0.717
	ADPKD	0.328	0.252
	Healthy volunteers	0.021	0.934
RHI	Both	-0.238	0.190
	ADPKD	0.006	0.983
	Healthy volunteers	-0.400	0.100
Homocysteinemia	Both	0.109	0.640
	ADPKD	0.272	0.479
	Healthy volunteers	0.215	0.441

4.3.8.4. Urine concentration of endogenous nitrite in ADPKD patients and healthy volunteers.

Urine concentration of endogenous nitrite was higher in healthy volunteers than in normotensive ADPKD patients with early stage disease (CKD1 and 2) and this difference was highly significant as shown in figure 4.19 (375.5 ± 198.4 Vs 216.0 ± 107.3 µmol/L, p<0.01).

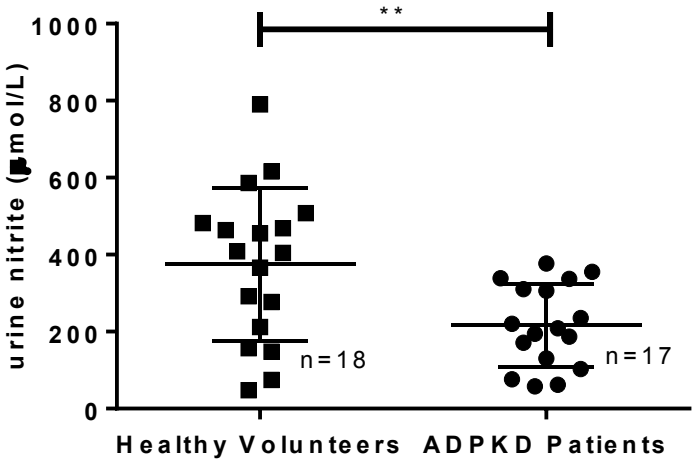


Figure 4.18: Urine nitrite concentration in healthy volunteers and normotensive ADPKD patients with early disease (**:p<0.05).

4.3.8.5. Relationship between endogenous urine nitrite and some cardiovascular risk factors in ADPKD patients and healthy volunteers.

The correlation between urine nitrite and eGFR as well as some cardiovascular risk factors are shown in table 4.13. There was no significant correlation with any of these factors in normotensive ADPKD patients with early disease. (p<0.05).

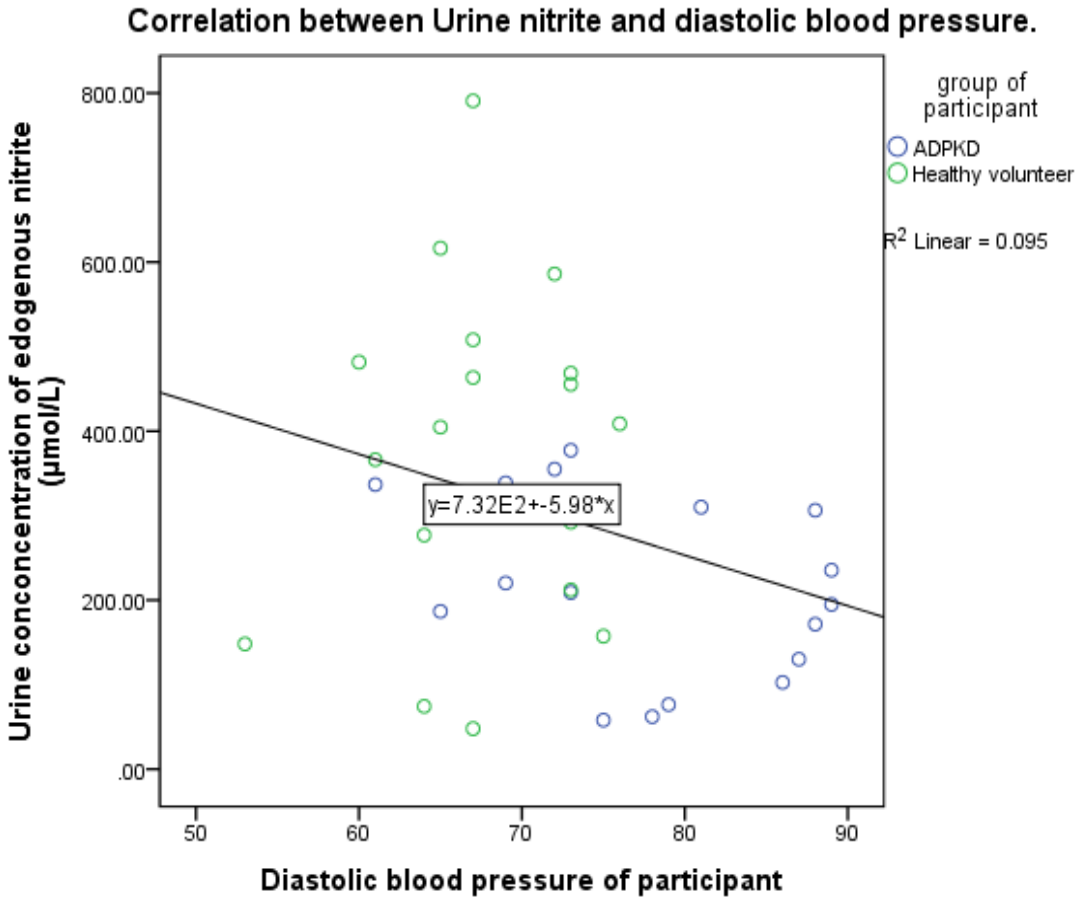
Table 4.13: Correlation between urine nitrite and cardiovascular risk factors as well as kidney function *(p value greater than 0.05 was considered not statistically significant)*.

Variables	Groups	Pearson correlation	P value
eGFR	Both	0.127	0.498
	ADPKD	0.302	0.239
	Healthy volunteers	-0.190	0.535
Proteinuria	Both	-0.193	0.306
	ADPKD	0.295	0.250
	Healthy volunteers	-0.055	0.857
ADMA	Both	0.057	0.749
	ADPKD	-0.132	0.627
	Healthy volunteers	0.167	0.508

4.3.8.6. Relationship between urine endogenous nitrite and diastolic blood pressure in ADPKD patients and healthy volunteers.

Figure 4.20 shows the correlation between urine endogenous nitrite and diastolic blood pressure in normotensive ADPKD with preserved kidney function as well as healthy volunteers. Nitric oxide decreased as blood pressure increased and this relationship was statistically significant ($r= -0.308$, $p<0.05$).

Figure 4.19: Urine nitrite and diastolic blood pressure in ADPKD patients with early disease and healthy volunteers.



Relationship between urine nitrite and diastolic blood pressure. There is a significant correlation between these two variable in healthy volunteers and normotensive ADPKD patients with preserved kidney function. (r=-0.308,p<0.05, ADPKD: Autosomal dominant polycystic kidney diseasse)

4.4 Discussion

In this prospective study of endothelial dysfunction in normotensive ADPKD patients with early stage disease (CKD stages 1 and 2), 8 isoprostane was not a significant biomarker of oxidative stress and asymmetric dimethyl arginine, matrix metalloproteinase 2 as well as PGE2 were not significant biomarkers of endothelial dysfunction. Conversely, the concentration of nitric oxide was significantly less in normotensive ADPKD patients with early stage disease when compared with healthy volunteers. In addition, nitric oxide concentration decreased as blood pressure increased in these normotensive participants. Concerning non-invasive assessment of endothelial dysfunction, I found no significant difference between healthy volunteers and normotensive ADPKD patients with early stage disease when ED was measured using EndoPAT 2000. In earlier studies, non-nephrotic range proteinuria which correlates with kidney function was reported in ADPKD patients (231, 232). However, there is paucity of reports on proteinuria and its correlation with ED in early stages of the disease. In my study, I found significantly higher proteinuria in this group. Similarly, the plasma homocysteine has been reported to be higher in ADPKD patients but there is paucity of data on its correlation with ED in early stage disease. I found no difference in plasma level of homocysteine between patients and controls. Furthermore, 24hour creatinine clearance had no correlation with eGFR and RHI in this study.

There were reports of 8 isoprostane as the gold standard biomarker of oxidative stress and there were also few reports of a significantly higher level of this biomarker in early stage disease ADPKD patients. However, most of the studies recruited participants with features that were known risk factors of oxidative stress. Conversely, I found no significant difference in plasma level of epi-$PGF_{2\alpha}$ (8 isoprostane) between patients and controls. I investigated this

biomarker in a selected population of early stage disease ADPKD patients. For instance, I excluded participants who were found to have higher level of C reactive protein. This is important as C reactive protein had previously been reported to be elevated in oxidative stress and ED (233).

In my study of ADMA as a biomarker of ED in ADPKD patients with early stage disease, I found no significant difference when compared with controls. Interestingly, I found significantly higher level of ADMA in patients when samples were stored without anti-oxidant. Earlier studies did not report storage of samples with anti-oxidant and that would not have been necessary as ADMA assay protocol did not come with such an instruction. Presumably, storage of samples with anti-oxidant significantly determines the result of plasma ADMA in ADPKD patients with early stage disease and healthy controls, especially, when participants were carefully selected to exclude known risk factors for ED. There was an earlier report which suggested an association between MMP2 and ED (187). Nagareddy and colleagues had suggested that MMP2 impaired endothelial function by uncoupling eNOS and that inhibition of MMP2 prevented the development of hypertension in an animal model of acquired systolic hypertension and insulin resistance. However, there was no statistically significant difference in endothelial dysfunction as measured by serum level of matrix metalloproteinase 2 (MMP2) between ADPKD patients with early stage disease and healthy controls. Also, I found no significant difference in urine concentration of PGE2 between normotensive ADPKD patients with early stage disease and healthy volunteers and there was no significant association with cardiovascular risk factors. This was at variance with the reports of Farmer and colleagues who had earlier suggested the possible role of PGE2 in endothelial function (234) and that of Tang et al who concluded that the impairment of PGE2-mediated relaxation may contribute

to endothelial dysfunction (235). Interestingly, almost all previous reports of PGE2 in ADPKD were limited to its effect of stimulating cytogenesis(196, 236).

Nitric oxide has a very short half-life due to its rapid metabolism. Therefore, the concentration of its metabolites (nitrite/nitrate) in body fluid had been reported to be sensitive markers of its activities (237) . I investigated nitrate/nitrite concentrations in blood and urine and found that serum nitrate/nitrite concentration was significantly reduced in patients than in controls and it correlated with DBP. Similarly, urine endogenous nitrite was significantly reduced in patients than in controls. Nitric oxide concentration correlated significantly with blood pressure in both serum and urine of healthy volunteers and normotensive ADPKD patients with early stage disease than healthy volunteers. These findings were consistent with earlier report by Wang and colleagues who concluded that ADPKD patients have defective nitric oxide generation (209).

Concerning non-invasive assessment of ED, I used the technique of finger plethysmography with the aid of EndoPAT machine and found no significant difference in RHI between healthy volunteers and normotensive ADPKD patients. There is paucity of data from similar population of patients using EndoPAT. This agreed with earlier report by Clausen et al(149). They investigated ED in 27 early disease ADPKD patients and concluded that there was no difference in flow associated dilatation of brachial artery between patients and controls. Vascular ultrasound was used in the non-invasive assessment of vascular reactivity in that study while finger plethysmography, which is not operator dependent was used in my study. Conversely, Kocaman and colleagues also used high resolution vascular ultrasound to assess ED in 15 hypertensive and 16 normotensive ADPKD patients with early disease. 16 patients with essential hypertension and 24 healthy volunteers were also recruited. They found significantly reduced FMD in both hypertensive and normotensive ADPKD patients with early

disease and concluded that there was significant ED in ADPKD patients with early disease (147). However, all hypertensive patients in that study continued their medications and the author admitted that as a limitation. Also, smokers were recruited and there was no mention of exclusion of participants found to have raised inflammatory markers. All these significant limitations were addressed in my study. Klawitter and colleagues suggested that ED was present in ADPKD patients, even in those with preserved kidney function but they did not provide details of features such as smoking status of participants as well as their drug history (199). Non-invasive study of endothelial dysfunction has been reported widely in the literature. However, most of the studies were carried out using the ultrasound machine and this has been reported to be highly observer dependent. There is a more portable way of investigating endothelial dysfunction non-invasively. This is done using a machine called EndoPAT. The details of how this machine works is as described earlier in chapter two. EndoPAT 2000 has been reported to detect endothelial dysfunction in some disease conditions. However, there are no reports on the use of this novel device in early stage disease ADPKD. In summary, most published studies of endothelial dysfunction in ADPKD patients involved the recruitment of participants with features which are known risk factors for ED. Also, there have been few reports on the concentration of nitric oxide in normotensive ADPKD patients with preserved kidney function. The finding of significantly decreased nitrite concentration in this study suggest that there might be subtle changes in the endothelium that are undetectable by finger plethysmography or that only selected vascular beds are affected.

CHAPTER FIVE

Investigations into endothelial dysfunction using immunohistochemistry.

In this chapter, I present the results of investigation into whether pkd2-/+ heterozygous mice exhibit endothelial dysfunction in mesenteric vessels and kidney compared with wildtype.

5.1 Introduction

This chapter aims to investigate expression of biomarkers of ED and oxidative stress using immunohistochemistry. These biomarkers are superoxide dismutase 2 (SOD2), hemeoxygenase 1 (HO1), matrix metalloproteinase 2 (MMP2) and 8 hydroxydeoxyguanosine (8OHdG). The experimental animals used for this study were obtained from Yale university, courtesy of Prof Somlo and the tissue fixation was by Fiona Wright, the histology technician, University of Sheffield.

5.2 Methods.

Details of animal model and handling were described in chapter 2.

5.3 Results

5.3.1 Semi-quantitative assessment of expression of superoxide dismutase (SOD2) in wildtype and Pkd2 -/+ heterozygous knockout mice.

Figure 5.1 shows SOD2 immunostaining in non-stressed (right) kidneys as well as ischaemic reperfusion injury (left) kidneys of heterozygous Pkd2 KO and wild type mice. When quantified as described in chapter 2, there was a statistically significant difference

in staining intensity score between wild type and heterozygous Pkd2 mice kidneys ($p<0.0001$).

Pkd2 mice showed reduced expression of SOD2.

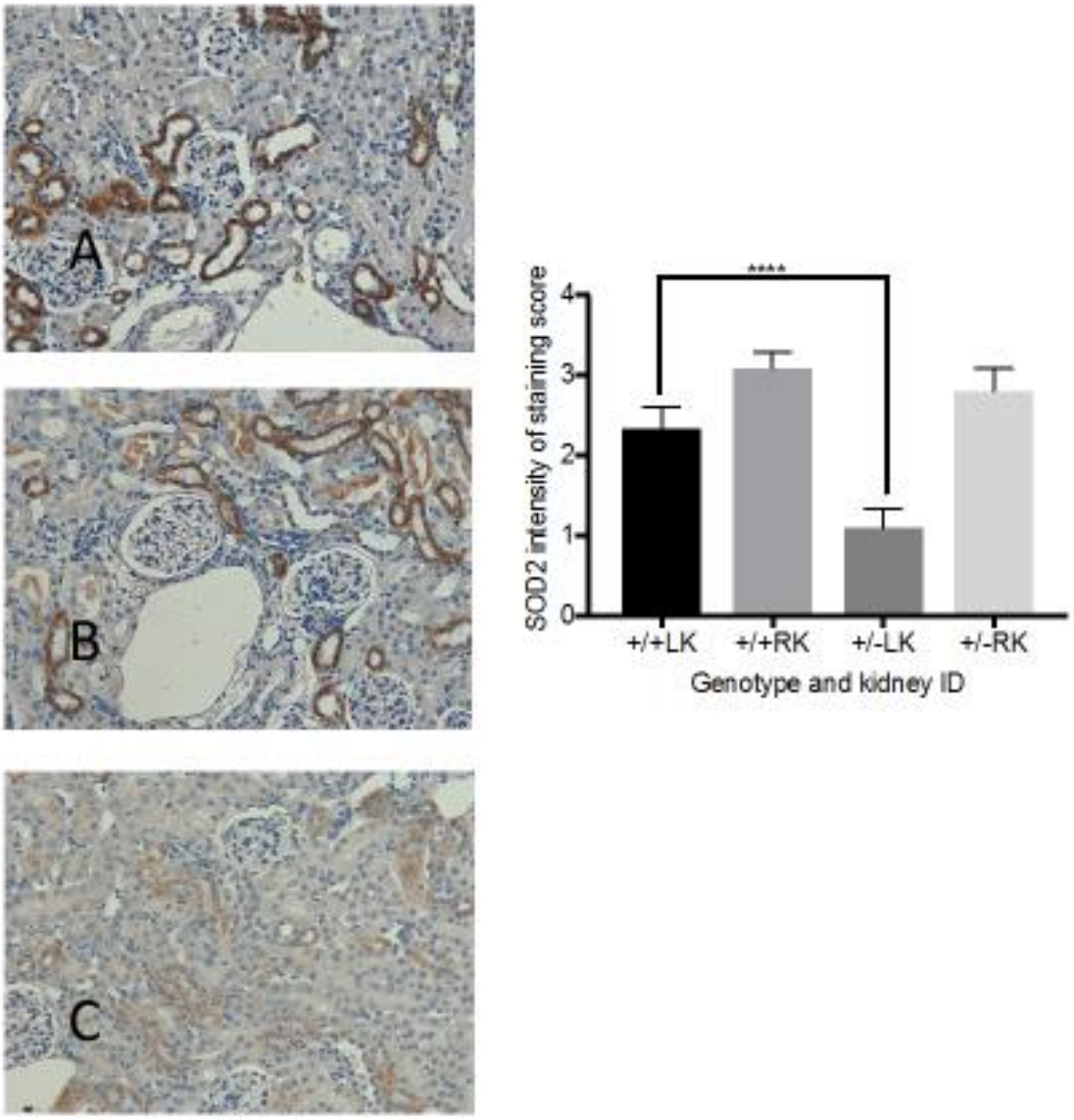


Figure 5.1. Super oxide dismutase 2 immunostaining of non-stressed (A) and ischaemia reperfusion injury (IRI) kidneys of wild type (B) and heterozygous Pkd2 (C) mice. (****=p<0.0001).

5.3.2 Heme oxygenase 1 immunostaining results.

Figure 5.2 shows heme oxygenase 1 (HO1) immunostaining in non-stressed as well as ischaemic reperfusion injury kidneys of wild type and heterozygous Pkd2 mice. When quantified, I found a greater level of HO1 staining in pkd2 heterozygous knockouts compared with wild type and this was statistically significant ($p<0.05$).

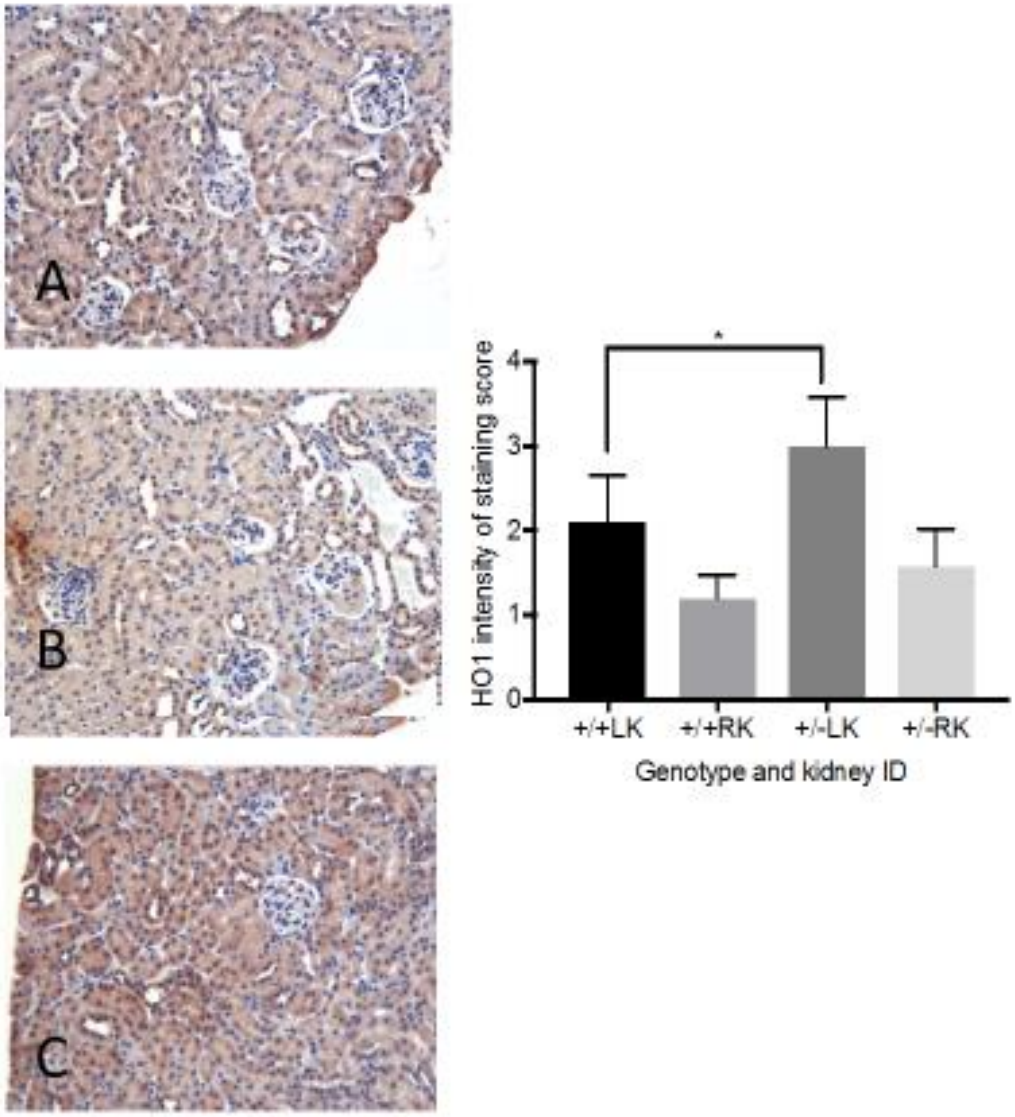


Figure 5.2 Heme oxygenase 1 immunostaining of non-stressed (A) and ischaemia reperfusion injury (IRI) kidneys of wild type (B) and heterozygous Pkd2 (C) mice. (*=p<0.05).

5.3.3 Hydroxyl deoxy guanosine (8 OHdG) immunostaining in mice mesenteric vessel and kidney tissue.

Figure 5.3 shows 8OHdG immunostaining in non-stressed as well as ischemic reperfusion injury kidney (IRI) kidneys of wild type and heterozygous Pkd2 mice. I observed staining in different parts of the kidney. However, I did not do a semi quantification of the staining intensity as there was much background staining and I ran out of time.

Figure 5.4 shows 8OHdG immunostaining in mesenteric vessels of wild type and heterozygous Pkd2 mice. Using one-way ANOVA, there was significant difference in the intensity of staining between wild type and heterozygous Pkd2 mice ($p<0.05$).

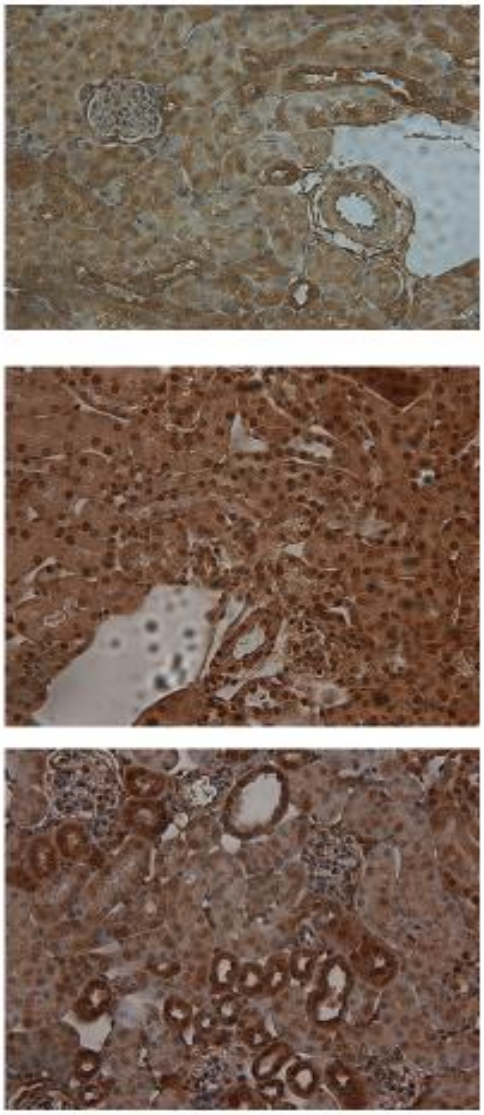


Figure 5.3 8 hydroxyl deoxyguanosine immunostaining of non-stressed (A) and ischaemia reperfusion injury (IRI) kidneys of wild type (B) and heterozygous Pkd2 (C) mice..

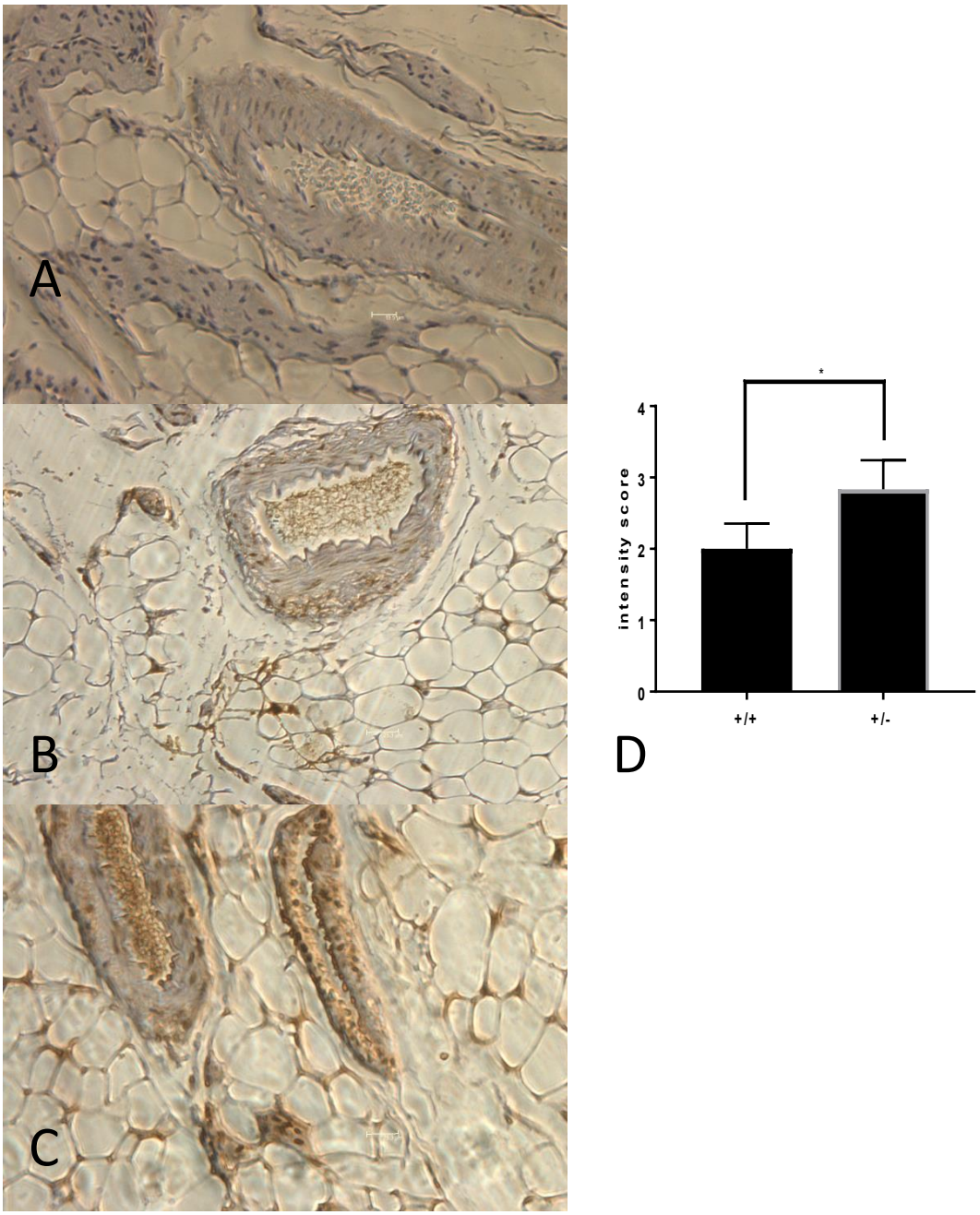


Figure 5.4. Negative control (A) as well as 8 hydroxyl deoxyguanosine immunostaining of WT (B) and heterozygous Pkd2 (C) mice mesenteric vessels. There was greater expression in heterozygous Pkd2 (D).

5.3.4 Matrix metalloproteinase 2 immunostaining of kidney tissue.

Figure 5.5 shows mouse on mouse matrix metalloproteinase 2 immunostaining of wild type and heterozygous Pkd2 mice as well as IgG negative immunostaining. I observed MMP2 protein expression, but I did not proceed with its quantification as a biomarker of endothelial dysfunction as the expression was predominantly extracellular.

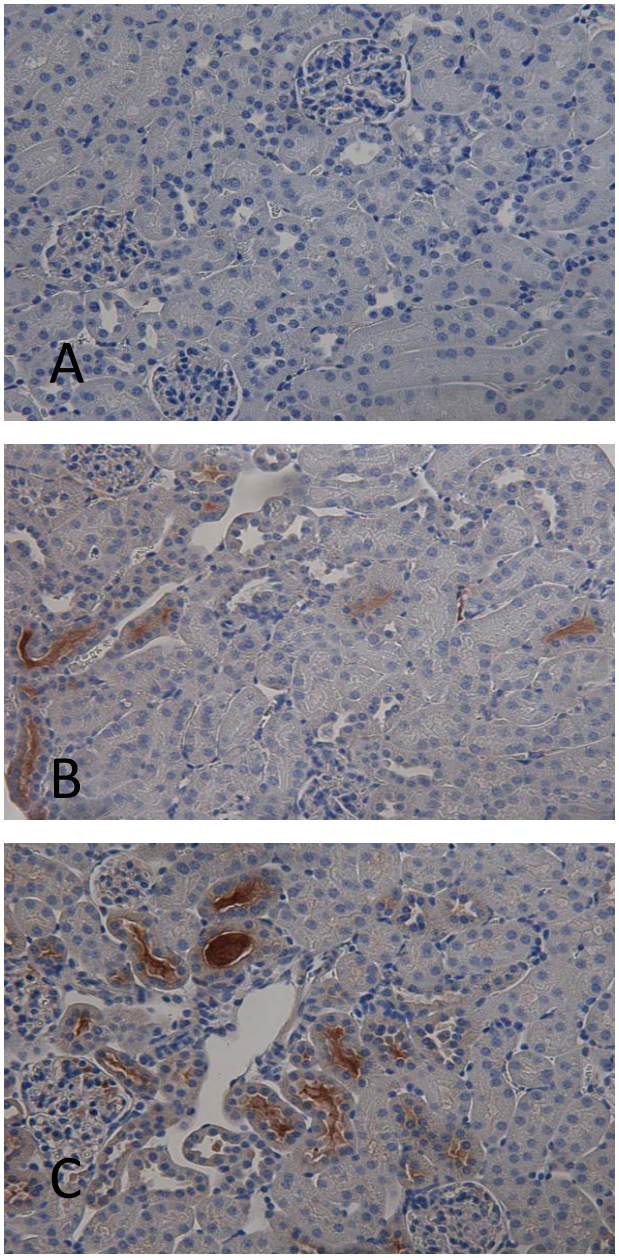


Figure 5.5 Negative control IgG (A) as well as matrix metalloproteinase 2 (MMP2) immunostaining of WT (B) and heterozygous Pkd2 (C) mice kidney tissue.

5.4 Discussion.

In this study, immunohistochemistry of three biomarkers of oxidative stress (8OHdG, SOD2 and HMOX1) and one novel biomarker of endothelial dysfunction (MMP2) was performed. There was significant oxidative stress in heterozygous Pkd2 knock out mice as evidenced by detection, localization and quantification of SOD2 and HO-1. Similarly, I detected increased expression of 8OHdG protein in Pkd2. However, MMP2 protein expression was not quantified as it was predominantly extracellular.

Superoxide anions (SOA) are the commonest ROS under physiologic condition and they are spontaneously produced around the electron rich mitochondrial membrane, the respiratory chain (238) and endoplasmic reticulum (239). Superoxide dismutase is an enzyme that catalyses the dismutation of SOA into hydrogen peroxide or molecular oxygen. In this study, we investigated the activity of one of its isotypes, superoxide dismutase 2 in kidney tissue of wild type as well as heterozygous Pkd2 KO mice using immunohistochemistry. Agharazi and colleagues had reported decreased SOD2 expression in CKD rats (240). Similarly, Krueger et al investigated SOD2 protein expression in CKD patients and they reported that lower expression of this protein was associated with better survival (241). However, very little is known about the activity of this enzyme in ADPKD and this is despite its reported therapeutic potential as a cardio protective agent. In this study, I observed lower expression of SOD2 in heterozygous Pkd2 mice and this was statistically significant when compared with wild type. This suggested a greater level of oxidative stress in Pkd2. In a related study, Menon et al reported lower serum SOD in hypertensive ADPKD patients with no graded difference across the CKD stages (170). Also, in a study of mice with cardiac specific SOD2 overexpression, Kang and colleagues observed supernormal cardiac function which was produced due to the ability

of this enzyme to effectively enhance mitochondrial function and subsequently promote metabolic vasodilation (242).

Furthermore, I detected significant greater expression of HO1 in heterozygous Pkd2. Heme oxygenase is an important enzyme for all organisms that depend on aerobic oxidation (243, 244). HO1, the inducible isoform of HO has been reported to be cytoprotective through its anti-inflammatory, antiproliferative and antiapoptotic effects (245). Reports about activities of HO1 in ADPKD is sparse. HO1 induction has beneficial effects and It has been reported as a potential therapeutic target in acute kidney disease (246). This beneficial effects of HO1 were partly attributed to some of its metabolites such as Iron, carbon monoxide and bile pigments. There is a paucity of information on tissue detection of this protein in non ADPKD as well as ADPKD kidneys. My data support the need for more investigations into role of HO1 as a therapeutic option in ADPKD.

The observed significant activity of SOD2 as a biomarker of oxidative stress is of interest when considered alongside that of HO1. Both biomarkers had previously been reported to be important biomarkers of oxidative stress in other disease conditions with very few reports in ADPKD patients. Interestingly, I observed that SOD2 is a better marker it terms of statistical strength. However, there is need for larger studies to test this suggestion. This becomes more important now that there is search for effective treatment options (170, 247).

Furthermore, I detected and localised 8OHdG in mesenteric vessels as well as kidney tissue of heterozygous Pkd2 mice. Single electron reduction in molecular oxygen leads to production of reactive oxygen species (ROS) which have beneficial effect in aerobic organisms as it is involved in cell survival, signalling pathway and protection. Conversely, accumulation of ROS produces deleterious effects (248). Repair process that follows ROS-mediated DNA damage

results in the formation of 8OHdG and it is the most recognised marker of oxidative stress when considering DNA damage products (174). However, there is paucity of data on its activity in ADPKD. In this thesis, I demonstrated that 8OHdG is expressed in kidney tissue of Pkd2 KO mice by using immunohistochemistry. This is in agreement with reported activity of 8OHdG in other disease processes(249, 250). While investigating the beneficial effect of kidney transplantation on evolution of oxidative stress in patients with end stage kidney disease, Cerrillos-Gutiérrez and colleagues reported significantly higher level of 8OHdG in patients with end stage kidney disease compared with healthy individuals. The level returned to normal within six months following kidney transplantation (251). Similarly, increased level of 8OHdG has been reported to be an independent predictor of all-cause mortality in hemodialysis patients (251). However, there are sparse reports about the activities 8OHdG as a biomarker of oxidative stress in ADPKD patients. My finding of increase activity of 8OHdG in Pkd2 suggest a further investigation into its activities in ADPKD.

Furthermore, I observed expression of MMP2 in PKd2 mice. Although quantification was not carried out as this expression was predominantly extracellular. Nagaredy el al investigated the role of MMP2 in endothelial function in animal model of acquired systolic hypertension and insulin resistance. They reported that increased activities of this protein produced impaired endothelial function and promoted hypertension (187). They suggested that MMP2 played a role in endothelial dysfunction probably through its ability to uncouple eNOS. This was novel as previous reports on MMP2 were mostly about its traditional role in enhancing digestion of extracellular structure. However, I did not find any evidence to suggest vascular expression of this protein in my study.

In conclusion, I found significant oxidative stress in heterozygous Pkd2 mice as evidenced by increase activities of SOD2, HO1 and 8OHdG. Also, MMP2 which was recently report to play a role in ED was not expressed on vascular tissue.

CHAPTER SIX.

GENERAL DISCUSSION.

In this thesis, I investigated 8 isoprostane, PGE2, SOD2, HO1, 8OHdG as biomarkers of oxidative stress in ADPKD. I also investigated ED by assessing concentrations of ADMA, MMP2 and nitrite/nitrate in blood and urine samples. These biomarkers were chosen because of their validated significance in other disease conditions as well as some reports from ADPKD studies. This thesis also highlights the use of an observer independent method of non-invasive assessment of ED using pulse arterial tonometry with the aid of EndoPAT machine. For the first time, not only were individuals with risk factors for ED excluded, those with ongoing inflammation as evident by raised highly selective CRP were also excluded from a study investigating endothelial dysfunction in early disease ADPKD patients.

There is growing interest into research aiming at identifying markers of cardiovascular risk in ADPKD patients. However, most previous studies involved recruitment of participants with hypertension, advanced kidney disease, obesity and smoking. These factors are known to be independently associated with endothelial dysfunction and are therefore major confounders (252, 253). Also, there is paucity of data on non- invasive investigation of ED in ADPKD patients. Available ones used ultrasound to assess FMD but this has been reported to be observer dependent (254). Also, some previous studies that were primarily designed to assess biomarkers of endothelial dysfunction investigated markers of oxidative stress only (170, 210). Thus, the focus of this research was to investigate endothelial dysfunction in normotensive ADPKD patients with early disease.

Firstly, I assessed the blood level of ADMA and 8 isoprostane as biomarkers of ED and oxidative stress respectively. The aim was to test the hypothesis that ED exists in ADPKD

patients. This initial part of the project was a retrospective study of blood samples of ADPKD and non-ADPKD patients at different stages of CKD, both early and late. There was significantly higher concentration of both biomarkers in ADPKD patients than controls. Similarly, non ADPKD patients had higher concentrations than controls. These agreed with most of the reports in the literature. However, this significance disappeared when a sub analysis of data was performed of patients with early disease among the participants in the retrospective study. There are postulations on the mechanisms by which cardiovascular disease results from endothelial dysfunction but there is no clear evidence of the proof of how this works in human (255). Cardiovascular features such as left ventricular hypertrophy that is found in about 4-40 % of ADPKD patients (256) are not only attributed to systemic hypertension but also to vascular disorders including endothelial dysfunction (76, 257). Also, there have been reports of increased serum biomarkers of oxidative stress in ADPKD patients (170) and 8 isoprostane have been described as the gold standard biomarker of oxidative stress (258). Menon and colleagues assayed plasma levels of 8 isoprostane in patients with ADPKD at different stages of the disease and in healthy controls. They concluded that oxidative stress was evident in ADPKD even with preserved kidney function (170). Conversely, I did not find any difference in the level of this biomarker in early ADPKD patients compared with controls. However, Menon and colleagues recruited 144 ADPKD patients with clearly defined characteristic features some of whom were participants in HALT-PKD trial (170) compared with smaller population size of my retrospective study. However, Menon and colleagues did not state if subjects with assayed plasma values that were not within the acceptable percentage of analysis (% B/Bo less than 20 or greater than 80) were excluded from their study. One of the groups in this retrospective study was made up of patients with chronic kidney disease from different primary causes (Non -ADPKD CKD). Chronic kidney

disease on its own has been reported variously to be a risk factor for cardiovascular disease and those on dialysis have 10-20 times increase mortality when compared with the general population. Worse still, individuals with CKD have a higher likelihood of dying from cardiovascular disease than progressing to end stage kidney disease (259, 260). These perhaps explained why the non-ADPKD CKD group also showed evidence of oxidative stress and ED. Lilitkarntakul and colleagues while assessing major determinants of endothelial dysfunction investigated the level of isoprostane in patients with CKD and healthy volunteers. They observed no significant difference between the concentration of 8 isoprostane in CKD patients at various stages of the disease and that of the healthy volunteers (261). However, smokers were not excluded from the population recruited into that study. There is paucity of reports on 8 isoprostane as a biomarker in ADPKD patients. However, there are reports of its elevation in CKD (262-265) with significant correlation with degree of progression of kidney disease (266, 267). Recently, Ramezani et al reported a significant decrease in plasma isoprostane level following kidney transplantation which suggests that this biomarker is a stronger indicator of the kidney state of health. Concerning ADMA, Klawitter and colleagues investigated endothelial dysfunction and oxidative stress in ADPKD and concluded that ADMA was higher in ADPKD patients than heathy controls even when the kidney function was preserved (199). I did not observe any difference in concentration between patients with early kidney disease and control. This biomarker, just like 8 isoprostane was only found to be higher in ADPKD patients when the participants were not separated into early or late disease. Again, there was no difference in concentration of ADMA between ADPKD and patients with non-ADPKD CKD when I considered data from those with early disease.

I next tested the hypothesis that there is increased biomarkers of oxidative stress and endothelial dysfunction in ADPKD patients in a prospective study involving recruitment of

early disease (eGFR≥60ml/min) ADPKD patients with normal blood pressure. I excluded, those with known risk factors for ED as well as those with evidence of raised inflammatory markers. In this prospective study group, I investigated ED by measuring levels of ADMA, MMP2 and nitrite/nitrate. To the best of my knowledge, this is the first ADPKD study where MMP2 was evaluated as a biomarker of ED. Reports about MMP2 were mostly within the context of its activities on extracellular matrix. Nagareddy et al had earlier reported that increased MMP2 activity impaired endothelial dysfunction (187). They suggested that this enzyme was involved in uncoupling eNOS with resultant decrease availability of NO. There is presently no data in ADPKD patients to test this postulated involvement of MMP2 in endothelial dysfunction. I found no difference in concentration of MMP2 between early disease ADPKD patients and healthy volunteers. However, I detected a significant decrease in blood and urine concentrations of nitrite/nitrate in normotensive ADPKD patients with early disease compared with healthy controls. This was in line with published evidence in the literature about total nitric oxide in ADPKD. Nitric oxide has been reported to be responsible for FMD of human peripheral conduit arteries (138). However, non-invasive assessment of ED in this group of patients showed no difference when compared with healthy volunteers. This suggests that there was subtle ED in early disease that could not be detected by pulse arterial tonometry. It is important to state here that the machine was used on some positive controls during this study and it detected ED in them as evident by low RHI. Using EndoPAT, Heffernan and colleagues compared ED in healthy volunteers with that of ADPKD patients with varying degree of renal function and concluded that healthy volunteers had significantly lower evidence of ED. However, confounders of ED like smoking were not excluded. The assessment of endothelial dysfunction using EndoPAT was described by Kuvin and colleagues as producing similar results with that seen when brachial ultrasound was used for the same purpose (268)

and the use of EndoPAT 2000 for the assessment of endothelial dysfunction was reported to predict CV events beyond Framingham risk score (156). I assessed EndoPAT study participants in a dedicated room in our clinical research facility. This was to ensure right environment in terms of lightings and noise control. Also, pre-and post-test time for the EndoPAT study was five minutes. This time limit have been reported to be optimal (269). In a study of 30 apparently healthy volunteers, Faizi and Kornmo et al compared the effect of varying the duration of occlusion (1.5, 3, 5 and 8 min) on endothelial response and they discovered that the effect was maximal at 5 minutes as the response at 8 minutes was not different for that at 5 minutes. My observation of lack of endothelial dysfunction in this prospective group was not surprising when considered alongside data from the sub analysis of the retrospective study in the earlier chapter.

Also, in the prospective study of normotensive early disease ADPKD and healthy volunteers, there was no difference in the blood level of PGE2 and 8 isoprostane as markers of oxidative stress. Also, there was no difference in the blood level of ADMA and MMP2 as biomarkers of endothelial dysfunction. However, the finding of higher level of proteinuria and blood pressure in the patients compare with the control could suggest subtle ongoing cardiovascular events in early disease that was not detectable by the EndoPAT as impaired RHI. This is suggested by correlation between proteinuria and RHI as well as between blood pressure and RHI.

I used numerous analysis to establish an association between ED as a dependent variable and several independent variables. The rationale for these analyses is to be as accurate as possible. Also, I used multiple regression as part of the analyses in order to fit a straight line

to several points. The rationale here is to learn more about the relationships between these variables and to detect probable outliers.

Finally, I tested the hypothesis that there is increased oxidative stress and endothelial dysfunction in ADPKD by using animal model of polycystic kidney disease. Heterozygous Pkd2 mice had earlier been reported to be more sensitive to IRI (228). I used a unilateral IRI mouse kidney model to assess oxidative stress and endothelial dysfunction and detected significant activities of SOD2, HO1, and 8OHdG in Pkd2 compared with wild type mice. There is paucity of data on the use of immunohistochemistry to describe the activities of SOD2 as a marker of oxidative stress. In this study, I observed decrease expression of SOD2 in heterozygous Pkd2 mice compared with wild type. Maser and colleague had reported a decrease in mRNA expression of superoxide dismutase in rats with progressive polycystic kidney disease and they suggested that decrease antioxidant enzyme protection was one of the general mechanisms in ADPKD disease progression (183). Tissue detection of SOD2 expression in this thesis supports the ongoing interest in its antioxidant as well as cardio protective properties. Furthermore, I observed increased expression of HO1 in heterozygous Pkd2 compared with wild type mice. Just like SOD2, HO1 has been reported to have cardio protective properties. I also assessed tissue level of 8-OHdG as marker of oxidative stress and found greater expression in the vascular endothelium of PKd 2 heterozygous knockout mice compared with that of wild type. This marker is a product of DNA damage and the most sensitive marker of oxidative damage (270). Aubaidy and Jelink compared 8 OHdG with markers of lipid peroxidation and antioxidant activity and concluded that it is a more sensitive marker of oxidative DNA damage (271). Also, I used mouse on mouse method of immunohistochemistry to detect MMP2 but I observed that this protein was expressed in the extracellular tissue and there was a trend of increased activity in heterozygous Pkd2 compared with wild type.

6.2 Limitations and suggestions for future work

There are some limitations of my studies. Firstly, the number of study subjects recruited was small. These findings will need to be replicated in a larger study with patients recruited from several centres. Secondly, I only studied eight selected biomarkers. This is a limitation as the use of a larger panel of biomarkers may increase sensitivity. Thirdly, several patients with unsuspected intercurrent illness had to be excluded from the final analysis therefore reducing statistical power. Fourth, only a very limited study by immunohistochemistry of a few proteins was conducted. Also, systolic blood pressure which is a major factor which might influence ED and oxidative stress was not reported in this study. Finally, the endoPAT study was newly established in our institution and should be validated against a gold standard technique with the inclusion of positive controls.

6.3 Conclusion:

ADPKD patients with early disease (normotension, eGFR>60) have higher diastolic blood pressure and microproteinuria compared to healthy controls. This correlates with reduced serum and urine nitrite concentrations suggesting a systemic defect in NO synthesis which was not detectable by finger plethysmography (EndoPAT 2000). Changes in several biomarkers of oxidative stress (8-isoprostane, ADMA) were absent in this cohort but were increased in a different patient cohort with hypertension and more advanced chronic kidney disease.

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Appendix 1: Copy of invitation letter

Dr Adebowale Adekoya
Clinical Research Fellow
Sheffield Kidney Institute
Northern General Hospital
Sheffield

Name

Address

Date

Dear Michael,

Invitation to participate in a research study

I would like to ask you to consider taking part in a research study entitled 'An Investigation in the role of endothelial dysfunction in Adult Polycystic Kidney Disease'. I am writing to you because as a patient with the above condition, in its early stages, you will enable us to help understand how the disease develops particularly in blood vessels. The study will be conducted by myself under the supervision of Professor Albert Ong and Dr Timothy Chico.

The study is trying to find out why patients with polycystic kidney disease develop high blood pressure when their kidneys are still working well. It will involve a one-off visit to the clinical research facility at the Royal Hallamshire Hospital. Once here we will scan your arm and check your blood pressure. We will also take a blood sample and urine samples.

Before you decide whether you want to take part, it is important that you understand why the research is being done. Please find enclosed an information sheet telling you more about why the study is being conducted and what it would involve for you.

Participation in this study is entirely voluntary. If you decide not to take part, you this will not affect the normal clinical services or treatment that you receive. If you participate in the study, all results would be treated in complete confidence.

If you have any questions or are interested in finding out more about participating in the study, please contact myself on 0114 271 4018.

Thank you for taking the time to read this letter.

Yours sincerely

Dr Adebowale Adekoya
Clinical Research Fellow

Appendix 2:

PARTICIPANT INFORMATION SHEET

(ADPKD Patient)

An investigation into the role of endothelial dysfunction in adult polycystic kidney disease

An invitation to take part in medical research
We would like to invite you to take part in our research study. Before you decide, it is important that you understand why the research is being done and what it will involve. This information sheet will help you in making the decision. Please take your time to read the following information and, if you wish, discuss it with friends, relatives or your doctor. If anything is not clear, or if you would like more information, please contact Dr Khamba on 0114 305 2056.

Who has reviewed this study?
All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your safety, rights, wellbeing and dignity. This study has been reviewed and approved by the South Yorkshire Ethics Committee.

What is the purpose of the study?
Patients with adult dominant polycystic kidney disease have cardiovascular complications which are present early in the disease for example high blood pressure. We know that there are several causes of high blood pressure. One of these causes is that the lining of blood vessels, the endothelium may not be working as efficiently and therefore be contributing to the high blood pressure. In patients with ADPKD we would like to study the lining of the blood vessels, called the endothelium, to see how well it is working. We will compare the results of patients with ADPKD and those who do not have the condition. If there is a significant difference, we will then aim to investigate this further and consider what medications may be useful in treating the endothelium and helping towards blood pressure control.

Why have I been invited?

You have not been singled out. All patients with adult dominant polycystic kidney disease in its early stages without hypertension have been invited to participate.

Do I have to take part?
No. Taking part is entirely voluntary. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. If you prefer not to take part, you do not have to give a reason and this will not affect the standard of care you receive. If you agree to take part, but later change your mind, you may withdraw at any time, without giving a reason. This will not affect your care in any way. Any identifiable information you have given will be destroyed, however we may use non-identifiable data collected up to your withdrawal.

What will happen to me if I take part?

If you agree to take part you will be invited to attend the Cardiovascular Biomedical Research Unit at Northern General Hospital, Sheffield.

On arrival to the research unit you will undergo the following:

- Interview to obtain medical history and general physical examination including blood pressure, height and weight (like a new patient clinic visit)
- Measurement of endothelium function (peripheral arterial tonometry using a system called EndoPAT). This will consist of having a blood pressure cuff attached to your forearm. It will be pumped up and kept inflated for five minutes. Your arm will also be scanned using ultrasound and the blood vessels identified. The diameter of the vessels will be measured. Once the blood pressure cuff is released, the blood vessel diameter will be measured again. The entire procedure is non-invasive and does not involve any ionising radiation. There may be some discomfort with the blood pressure cuff inflated but this will settle once the pressure is released. On very rare occasions some patients may find the procedure painful and can cause bruising. If you feel this is the case, then you can choose to withdraw from the study at any point.
- Blood will also be taken to check your kidney function, various heart disease markers e.g. cholesterol, markers of endothelial function and for DNA analysis. This will be a total volume of up to 100ml of blood.
- Urine will be collected for 24 hours prior to arrival and on the day and sent for analysis

The entire visit should last about 2 hours and will be conducted by a qualified doctor.

What will I have to do?

You should continue to do everything as normal. The day before we will ask you collect urine your urine for 24 hours and bring it to the lab. Bottles will be supplied. On the day of the visit we will ask to refrain from smoking and heavy exercise. The night before the visit we will ask you to fast from midnight Also on the day of the visit you should not take your medication until after the study.

What are the possible disadvantages and risks of taking part?
There may be some discomfort from the blood sampling which may cause a bruise but usually settles down within a few days. Any new health problems identified will be communicated with the participant and referred to the respective general practitioner or relevant medical department for follow up according to NHS protocols.

What are the side effects of any treatment received when taking part?

There are no treatments given during this study and therefore we expect no side effects to arise as a result of this. During the study, you will be given a medication called Glyceryl Tri Nitrate (GTN). This medication is given as a single dose, which is placed under the tongue to dissolve. This can cause flushing, headache and low blood pressure. Its effects are short term (less than an hour) and completely resolve by the time the study visit has concluded.

What are the possible benefits of taking part?

We cannot promise the study will help you but the information we get from this study will help improve the treatment of people with Adult Dominant Polycystic Kidney Disease.

What happens when the research study stops?

You will continue to get care from your normal medical teams. Your normal treatment will not be affected. You may be asked to attend for further study depending on the results of this study.

What about confidentiality?

All the information that is collected about you during the research will be kept strictly confidential. If you consent to taking part in the research study your medical records may be inspected by members of the research team (including a research nurse) for the purpose of analysing the results and ensuring their accuracy. They may also be looked at by the regulatory authorities or ethics committee to check that the study is being carried out correctly. However, all information that is collected about you will be kept strictly confidential and your name will not be disclosed outside of the hospital. With your permission, given by signing the consent form, we will tell your GP that you are involved in the study. When the results are published, no names will be used, and it will not be possible to identify anyone who has taken part.

What will happen to the results of the research study?

The information will be used to see whether there is difference in the endothelium function of patients with ADPKD compared to those who do not have ADPKD. If there is a difference, then this will form the basis of further studies to further investigate these differences.

We hope that the results of the study will be published in a scientific journal/s within the next year. We would like to emphasise that it will not be possible to identify you from any publication or report arising from this study. If you would like a copy of the research report, we can send this to you.

Who is organizing and funding the research?
This study is being run by Prof A C Ong and sponsored by Sheffield Teaching Hospitals NHS Trust. Funding has been obtained from National Institute of Health Research.

What if I wish to complain about the way in which this study has been conducted?

If you have a concern about any aspect of the study, please contact either the Principal Investigator:
Dr Adekoya Tel: 0114 271 3339 Sheffield Teaching Hospitals

If you remain unhappy and wish to make a formal complaint about any aspect of the study or how you have been treated during the study, the normal Sheffield Teaching Hospitals complaints procedure is available to you and are not compromised in any way because you have taken part in a research study. Please contact the following:

Patient Services Team Tel: 0114 2712400 Sheffield Teaching Hospitals

What do I do now?
Now that you have read the information leaflet, if you are happy to participate, please complete the consent form and return it, either in the pre-paid envelope provided or to a member of the research team. A member of the research team will then contact you with details of your appointments and further care. If you would like to discuss this information with your family, friends or GP or you would like to contact me or the research nurse to discuss this study further, please do. My contact details are given above. If you do not wish to take part, your clinical care will not be affected in any way.

Thank you

Dr Adebowale Adekoya
Clinical Research Fellow
Sheffield Teaching Hospitals NHS Trust

Appendix 3: Copy of consent form

Centre: Sheffield
Study Number: STH15983
Patient Identification Number for this trial:

CONSENT FORM

The Role of Endothelial Dysfunction in Adult Polycystic Kidney Disease
Prof A C Ong, Dr Chico and Dr Adekoya

		Please initial box
1.	I confirm that I have read and understand the information sheet dated V1.0 (version 14 January 2011) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2.	I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐
3.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from NIHR, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	☐
4.	I agree that samples taken during this study will be stored in the Bio repository at the Sheffield Kidney Institute and can be used for further research and analysis	☐
5.	I agree to my GP being informed of my participation in the study.	☐
6.	I agree to take part in the above study.	☐

Name of Patient	Date	Signature
Name of Person taking consent	Date	Signature

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

Appendix 4: PROTOCOL FOR BLOOD COLLECTION (STH 15983 PROJECT).

(Prof Albert Ong and Tim Chico's group)

- ✓ Patients have come fasting
- ✓ They have also come with 2 containers of urine (collect this and put in the ICE box provided, please)
- ✓ Take the specified amount of blood into the corresponding vacutainer (sample bottles)

Vacutainer (cap colour and content) Volume of blood to be taken

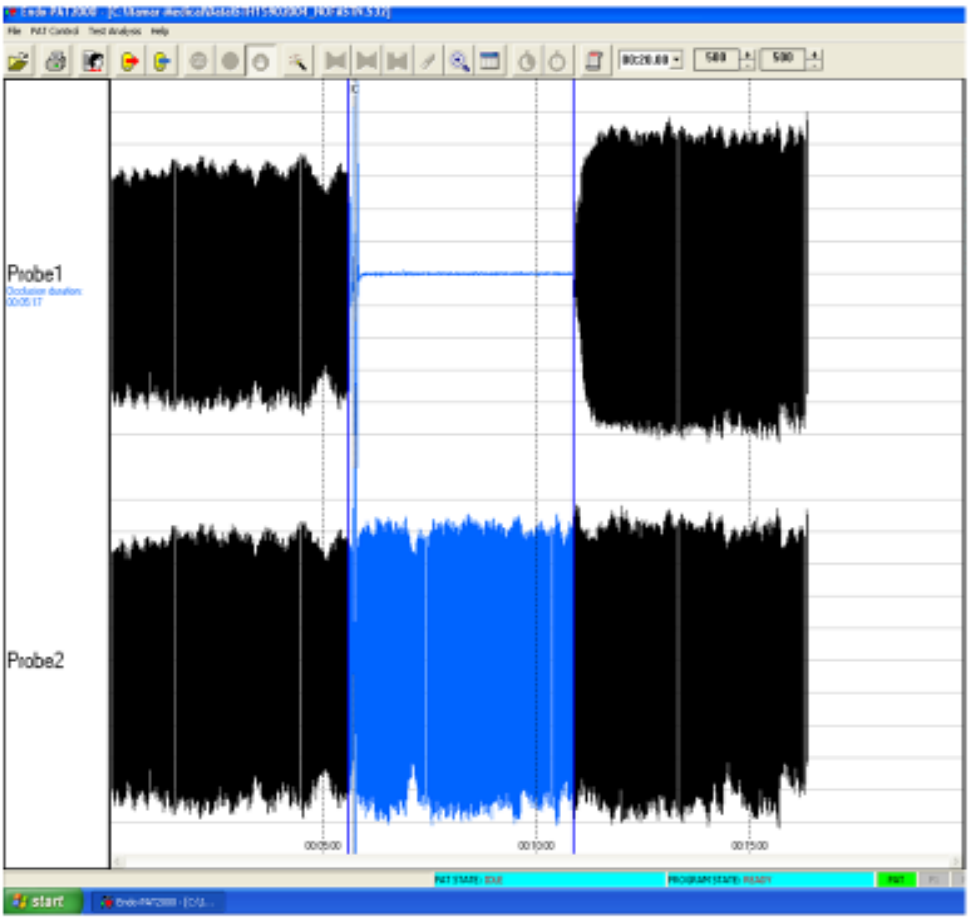
- Light green or Brown coloured top (contains no anticoagulant) ……. take 4ml x 2
- Lavender coloured top (contains K2EDTA) ……take 4mlx2
- Grey coloured top (contains Na fluoride and K oxalate) ……. take 6ml x 2

- ✓ Each one of the 3 pairs will go to the STH lab (i.e. one light green/brown top, one lavender top and one grey top) while others will be taken to the Lab on K floor by Dr Adekoya
- ✓ Patient investigation forms have already been completed and given handed over to them before coming to you for venepuncture.
- ✓ Please leave the samples going to K floor in the ICE box provided.

Vacutainer (cap colour and content) TEST

- Light green or Brown coloured top (no anticoagulant) …U&E, Ca, Po4, Lipids, albumin
- Lavender coloured top (contains K2EDTA) ……Homocysteine, FBC
- Grey coloured top (contains Na fluoride and K oxalate) ……Fasting blood glucose

Appendix 5: EndoPAT tracing



Appendix 6: Good Clinical Practice certificate

NHS
National Institute for Health Research
Clinical Research Network

Certificate of Completion

ADEBOWALE ADEKOYA

has completed

Introduction to Good Clinical Practice (GCP) e-learning course

A practical guide to ethical and scientific quality standards in clinical research

on 11 March 2013

Modules completed

Introduction to Research in the NHS
Good Clinical Practice and Standards in Research
Study Set-up and Responsibilities
The Process of Informed Consent
Data Collection and Documentation
Safety Reporting
Summary

Appendix 7: Research approval

Ref: STH15983 MH/JC

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

15 October 2013

Professor Albert Ong
Professor of Renal Medicine and Honorary Consultant Nephrologist
Sheffield Kidney Institute
Northern General Hospital
Sheffield
S5 7AU

Dear Dr Ong,

Substantial Amendment
Letter of Continued NHS permission

STH ref:	**STH15983**
NIHR CSP ref:	**71964**
REC ref:	**11/YH/0026**
MHRA ref:	**CTA no.: NA** **EudraCT no.: NA**
Study title:	**To investigate the role of endothelial dysfunction in adult dominant polycystic kidney disease.**
Principal Investigator:	**Dr Albert Ong, Sheffield Teaching Hospitals NHS FT**
Sponsor:	**Sheffield Teaching Hospitals NHS FT**
Funder:	**NIHR Research Fellowship**
Amendment ref:	**Substantial Amendment 1**

Thank you for submitting the following documents:

Document	***Version/date***
North East REC favourable opinion of amendment	26 Sep 13
Protocol	V3.0, 26 Apr 13
Participant Information Sheet: Healthy Volunteer	V2.0, 03 Jun 13
Participant Information Sheet: Patient	V2.0, 03 Jun 13
Consent Form	V2.0, 03 Jun 13
Letter of Invitation to participant: Patient	V2.0, 03 Jun 13
Letter of Invitation to participant: Healthy Volunteer	V2.0, 03 Jun 13
Investigator CV	Undated
GP Information Sheets	V2.0, 03 Jun 13
Notice of Substantial Amendment (non-CTIMP)	SA1, 03 Jun 13

These have been reviewed by the Research Department who have no objection to the amendment and can confirm continued NHS permission for the study at STH.

Yours sincerely

Professor S Heller
Director of R&D, Sheffield Teaching Hospitals NHS Foundation Trust
Telephone +44 (0) 114 22 65934
Fax +44 (0) 114 22 65937

cc. Louese Dunn, CRF