Mineralocorticoids and sodium in chronic kidney disease – regulation and cardiovascular implications.

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Author's declaration

The work presented in this thesis was that of the author and her supervisors, Professor Alan Jardine and Professor John Connell. All experimental work was carried out by the author unless otherwise stated.

I declare that this thesis has been composed by myself and is a record of work performed by myself. It has not been previously submitted for a higher degree.

Emily Pamela McQuarrie

December 2011

Definitions/Abbreviations

11β-HSD	11 β-hydroxysteroid dehydrogenase
24h QP	24h quantified total proteinuria
ACE	Angiotensin converting enzyme
ACEi	Angiotensin converting enzyme inhibitor
ACR	Albumin to creatinine ratio
ACTH	Adrenocorticotrophic hormone
AIx	Augmentation index
AIx@75bpm	Augmentation index normalised to 75bpm
Aldo	Aldosterone
ANOVA	Analysis of variance
APKD	Adult polycystic kidney disease
ARB	Angiotensin receptor blocker
ARR	Aldosterone to renin ratio
BBl	Beta blocker therapy
BMI	Body mass index
BNP	Brain natriuretic peptide
BSA	Body surface area
C&G	Cockcroft and Gault
C1	Collagen 1
CCB	Calcium channel blocker
Chol:HDL	Cholesterol to high density lipoprotein ratio
CKD	Chronic kidney disease
CMR	Cardiac magnetic resonance imaging
Cort metab	Cortisol metabolites
Cr	Creatinine
CrCl	Creatinine clearance
CRH	Corticotrophin releasing hormone
CRP	C-reactive protein
CT	Threshold cycle
CV	Cardiovascular
DBP	Diastolic blood pressure
DMN	Diabetic nephropathy
DOC	Deoxycorticosterone
DOCA	Deoxycorticosterone acetate
E	Cortisone
ECG	Electrocardiogram
Echo	Echocardiogram
ED	End diastole

EDV	End diastolic volume
eGFR	Estimated glomerular filtration rate
EH	Essential hypertension
ELISA	Enzyme linked immunosorbent assay
ENaC	Epithelial sodium channel
ERK	Extracellular signal-related kinases
ESRD	End stage renal disease
ESV	End systolic volume
F	Cortisol
FBS	Fetal bovine serum
FENa	Fractional excretion of sodium
FMD	Flow mediated dilatation
GM-CSF	Granulocyte macrophage colony stimulating factor
GN	Glomerulonephritis
Hb	Haemoglobin
HDL	High density lipoprotein
HK-2	Human kidney 2 cells
HLA	Horizontal long axis
HR	Heart rate
Ig	Immunoglobulin
IgAN	IgA nephropathy
IHD	Ischaemic heart disease
IL	Interleukin
K	Potassium
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
LV	Left ventricle
LVMI	Left ventricular mass index
LVOT	Left ventricular outflow tract
LVSD	Left ventricular systolic dysfunction
MAP	Mean arterial pressure
MC	Mineralocorticoid
MC	Myocardial
MDRD	Modification of diet in renal disease
MGN	Membranous nephropathy
mmHg	Millimetres of mercury
MR	Mineralocorticoid receptor
MRB	Mineralocorticoid receptor blocker
MRI	Magnetic resonance imaging
NO	Nitric oxide
PAC	Plasma aldosterone concentration

PAT	Peripheral artery tone
PCR	Polymerase chain reaction
PO_4	Phosphate
PP	Pulse pressure
PRC	Plasma renin concentration
PTH	Parathyroid hormone
PWA	Pulse wave analysis
PWV	Pulse wave velocity
RAAS	Renin-angiotensin-aldosterone system
REC	Research ethics committee
RF	Radiofrequency
RHI	Reactive hyperaemia index
RIA	Radioimmunoassay
ROMK	Renal outer medullary potassium channel
RRT	Renal replacement therapy
SA	Short axis
SBP	Systolic blood pressure
Statin	HMG Co-A reductase inhibitor
TG	Triglyceride
TGFβ	Transforming growth factor beta
THAldo	Tetrahydroaldosterone
THDOC	Tetrahydrodeoxycorticosterone
UNa	24h Urinary sodium excretion
UNa:Cr	Urinary sodium to creatinine ratio
uPCR	Urinary protein to creatinine ratio
UPot	Urinary potassium excretion
UPr	Urinary protein excretion
VLA	Vertical long axis
WNK	With-no-K[Lys] protein kinases

Summary

Chronic kidney disease is common and associated with an elevated cardiovascular risk, as well as the long-term risk of renal failure. At present, therapeutic approaches to managing chronic kidney disease (CKD) do not fully reverse these risks. This has led to study of the determinants of pathological outcomes in these patients, with the hope of further therapeutic interventions to reduce these risks.

Mineralocorticoids, predominantly aldosterone, are produced by the adrenal cortex and have a vital role in maintaining sodium status and blood pressure. However, high levels of aldosterone in humans are known to produce an adverse phenotype of hypertension and a disproportionately elevated cardiovascular risk. Furthermore, in animal models of renal failure, elevated aldosterone levels stimulate renal damage, in the presence of a high sodium milieu. These laboratory findings have been translated to provide a basis for several short-term follow-up clinical trials looking at the impact of non-genomic non-natriuretic doses of mineralocorticoid receptor inhibition in patients with chronic kidney disease. These studies have shown a reduction in proteinuria, often independent of decline in blood pressure. However, there is a paucity of baseline physiological data relating to the normal regulation of mineralocorticoid synthesis and action in chronic kidney disease. The response of the adrenal cortex to renal failure is not understood. Is mineralocorticoid synthesis regulated in the usual way? Are the stimulators of mineralocorticoid production and release affected by uraemia? Is dietary sodium intake associated with steroid status and adverse outcomes in humans?

The hypothesis of this thesis was that the renin-angiotensin-aldosterone system is inappropriately activated in patients with chronic kidney disease. Secondly, that high levels of mineralocorticoids are associated with adverse end-organ damage including proteinuria excretion, left ventricular hypertrophy, endothelial dysfunction, elevated pulse wave velocity and markers of renal fibrosis. Furthermore, that these deleterious effects are associated with sodium status and that an elevated dietary sodium intake is independently associated with increased renal and cardiovascular risk. In order to test these hypotheses, 70 patients with CKD and 30 patients with essential hypertension (EH) were recruited and underwent detailed clinical and biochemical phenotyping. This included 24 hour urinary steroid metabolite analysis, plasma renin and aldosterone measurement, cardiac magnetic resonance imaging, carotid-femoral pulse wave velocity and assessment of endothelial function.

It was shown that levels of the main mineralocorticoids (MC) (aldosterone and deoxycorticosterone) are not elevated in patients with CKD, as compared with patients with essential hypertension (EH). However, the determinants of levels of MC excretion differed between the two conditions. In CKD, excretion of MC metabolites was directly proportional to excretion of urinary sodium. A high urinary sodium (a marker of dietary sodium intake) was associated with a higher excretion of tetrahydroaldosterone (THALDO - the main aldosterone metabolite). In patients with EH, no relationship was seen between urinary steroid excretion and urinary sodium excretion. This is a novel relationship between the kidney and adrenal gland which questions the conventional wisdom that the adrenal cortex is unaffected by uraemia and prompts further study into the regulation of steroid synthesis in CKD.

Furthermore, it was shown for the first time that 24h excretion of tetrahydrodeoxycorticosterone (THDOC) is an independent predictor of left ventricular mass index and that THALDO is an independent predictor of proteinuria excretion – demonstrating a relationship between mineralocorticoids and two of the main predictors of mortality in CKD. An interaction between sodium, MCs and these two features was also demonstrated.

No association between levels of mineralocorticoids and vascular function was seen. Urinary 24 hour excretion of sodium was significantly associated with endothelial dysfunction in patients with CKD and pulse wave velocity in patients with essential hypertension.

Retrospective data analysis further confirmed an association between a high dietary sodium intake and adverse outcomes. In a study of 498 patients with CKD and a median follow-up of 7 years, an elevated 24h urinary sodium to creatinine ratio was shown to be associated with an increased risk of death. There was however no independent association with renal progression or requirement for renal replacement therapy. This is the first time that sodium intake has been clearly linked to adverse outcomes in patients with CKD.

Lastly, laboratory work demonstrated that steroid stimulation (aldosterone or cortisol) of human proximal tubular cells resulted in increased collagen 1 gene expression, but only in the context of a high sodium environment. Collagen 1 is deposited in renal interstitial fibrosis. This effect was inhibited by MR blockade, further expanding on the potential role

of steroids in the progression of CKD and again confirming the relationship between salt and steroids.

In conclusion, in this thesis it has been demonstrated that production of MCs in patients with CKD is closely associated with urinary sodium excretion (a surrogate for dietary sodium intake). This relationship is novel and not seen in patients with essential hypertension. It suggests that the response of the adrenal cortex in the context of uraemia is altered. Moreover, levels of mineralocorticoids are independently associated with left ventricular mass index and proteinuria excretion, both significant predictors of mortality, in patients with CKD. Dietary sodium intake has been shown to be an independent predictor of mortality and laboratory studies have demonstrated that mineralocorticoid receptor binding in a high sodium environment is associated with collagen 1 gene upreguation. These findings have important implications for the role of adequate renin-angiotensin-aldosterone blockade in patients with CKD and suggest that the addition of a mineralocorticoid receptor blocker and dietary sodium restriction should be advocated.

1 Chapter One - Introduction

1.1 General introduction

In this thesis, hypotheses relating to adrenal steroid metabolism and function in the context of chronic kidney disease will be addressed. The relationship between steroid levels and negative prognostic features including left ventricular hypertrophy, proteinuria, pulse wave velocity, endothelial dysfunction and inflammatory marker production will be examined. The role of sodium in pathophysiological outcomes, both as an independent feature and in the context of steroid levels will also be discussed.

In this introduction, the burden of chronic kidney disease and current therapeutic approaches will be discussed in section 1.2. Sodium homeostasis and the role of sodium in blood pressure control are discussed in section 1.3, in conjunction with evidence that a high sodium intake has adverse consequences. Adrenal steroid production (section 1.4) and consequences of mineralocorticoid excess in humans (section 1.5); in vivo (section 1.6) and in vitro (section 1.7) are further discussed. Section 1.8 describes left ventricular abnormalities in CKD- measurement and determinants; whilst section 1.9 describes abnormalities in the vasculature. Section 1.10 describes the aims of the thesis and section 1.11 summarises the hypotheses generated.

The influences of many systems on the body's electrolyte (sodium and potassium) status results in equilibrium, the disturbance of which would alter electrolyte and blood pressure homeostasis, often with clinically significant consequences. Prominent among these regulating systems are a number of aspects of corticosteroid metabolism.

1. Aldosterone is a product of the adrenal zona glomerulosa. It acts on sodium/potassium transporting epithelia via a nuclear receptor (the mineralocorticoid receptor: MR) and promotes sodium ion excretion in exchange for potassium and hydrogen ions, as well as having direct actions on other tissues. Excess secretion results in hypokalaemia, increased exchangeable sodium, metabolic alkalosis and hypertension. This is further discussed in sections 1.4 to 1.7.

2. Deoxycorticosterone (DOC) is principally a product of the adrenal zona fasciculata and is capable of acting as a mineralocorticoid. In normal human subjects, its secretion rate and plasma concentration are very low and little is known of its role, although it can become more abundant in some rare genetic diseases. It has frequently been used experimentally to model mineralocorticoid hypertension. New information on its

metabolism may alter assessment of its role. This is expanded upon in sections 1.4 and 1.6.3.

3. Cortisol exerts a potent influence on blood pressure by a variety of mechanisms Most relevant to this study, it is potentially a potent mineralocorticoid, binding with high affinity to the MR. This is normally prevented by the enzyme, 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) which oxidises cortisol to inactive cortisone. Genetic disruption of the gene for the enzyme leads to a severe mineralocorticoid hypertension. Other important indices used in this study are the levels of cortisol, cortisone, their respective metabolites and the relation between them (see sections 1.4.7, 1.4.8, 1.4.11).

1.2 CKD

1.2.1 Chronic kidney disease: risk, prevalence and consequence

Chronic kidney disease (CKD) is common, being present in 5-10% of the population(1;2) (Figure 1a), increasing markedly with age. Patients with CKD are at risk of progression to end stage renal disease (ESRD), requiring dialysis or kidney transplantation. Once patients have developed ESRD, their cardiovascular mortality is increased by 20-100 fold that of age matched individuals from the general population(3) (Figure 1b). Furthermore, provision of dialysis treatment costs approximately £30-35,000 per patient annually(4). Renal transplantation is a successful treatment of ESRD but requires a suitable kidney donor and only one third of patients with ESRD will be fit enough to undergo the renal transplant operation. There is a shortage of cadaveric organs with each patient waiting approximately 3 years prior to receiving a suitable organ. Therefore prevention of progression of CKD is desirable for individuals as well as for society.

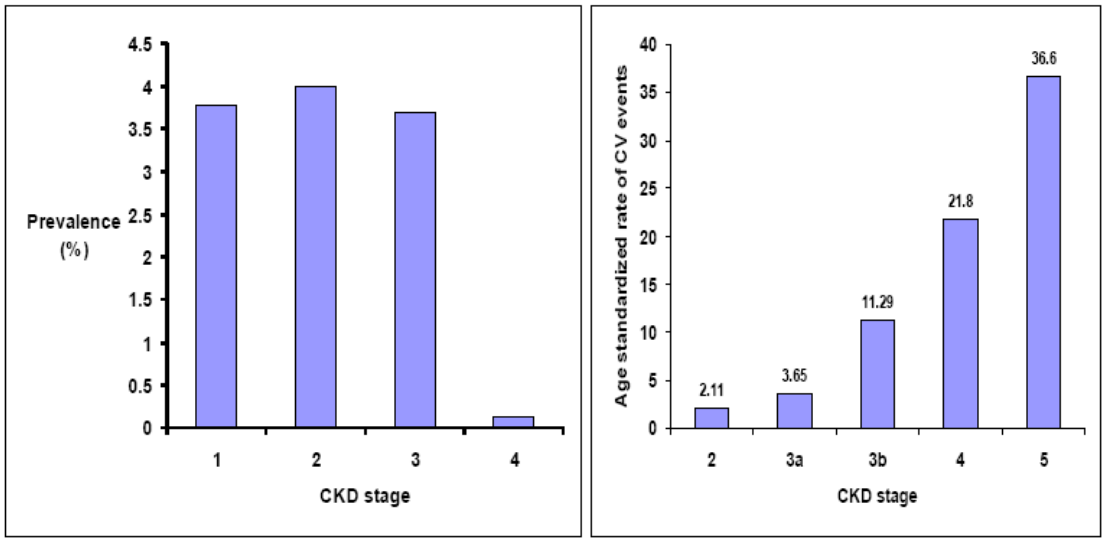


Figure 1-1: Chronic kidney disease prevalence and association with cardiovascular events.

A - Population prevalence of chronic kidney disease by stage. Data derived from Northern Europe and North America (2). B - Age standardised rate of cardiovascular events according to stage of chronic kidney disease (5).

The increased cardiovascular risk associated with CKD, not yet treated with dialysis, may reflect a clustering of cardiovascular risk factors (smoking, hypertension, hyperlipidaemia) but likely also relates to factors specific to kidney disease such as endothelial dysfunction, hyperhomocysteinaemia and oxidative stress(6). The presence of proteinuria is an additional independent risk factor for cardiovascular disease(7), even within the normal range(8). It may reflect glomerular dysfunction or endothelial dysfunction at the glomerular basement membrane.

1.2.2 Staging of chronic kidney disease

The stages of CKD are based on the glomerular filtration rate (measured or estimated). There are five stages, with stage 1 patients having normal excretory function and stage 5 being severely impaired renal function or the requirement for renal replacement therapy. The addition of a p suffix relates to the presence of significant proteinuria (>1g/d)(9).

1.2.3 Proteinuria

Proteinuria and level of renal function at presentation are the two best independent predictors of outcome in patients with chronic kidney disease (CKD)(10-13). This is true in patients with primary renal disease(14;15) and also in patients with diabetic nephropathy(16;17). Proteinuria is also a predictor of mortality in the general population (18) as well as in patients with CKD(7). Reducing proteinuria retards progression of renal disease(14;19).

Proteinuria is a largely a reflection of increased glomerular permeability secondary to disease, but can also be directly nephrotoxic. The toxic effects of proteinuria are mediated at the glomerulus and in the proximal tubule. In vitro, proximal tubular cells stimulated with serum proteins (albumin, IgG, transferrin) produce a number of profibrotic and pro-inflammatory markers at the basolateral membrane including endothelin and IL-8 (20). Furthermore, local tubular cells may be overloaded with filtered plasma proteins and signal for the recruitment of local macrophages. High molecular weight proteinuria has also been associated with proximal tubular cell apoptosis(21).

1.2.4 Blood pressure as a risk factor for progression in CKD

Multiple studies have demonstrated that control of blood pressure is an important prognostic indicator in terms of risk of progression of CKD(22). These findings have been shown in patients with diabetic nephropathy(23) and in patients with primary renal diseases(24). The optimal level of blood pressure control varies depending on the severity of proteinuria excretion. Effects are likely to be mediated via a limitation of glomerular barotrauma and endothelial dysfunction.

1.2.5 Interstitial fibrosis

The degree of interstitial fibrosis is the best histological correlate of risk of progressive renal disease(25). Whether it arises as a consequence of proteinuria being toxic to the proximal tubule, triggering inflammation and fibrosis, or more generalised renal ischaemia and glomerular hyperfiltration remains unclear(26).

1.2.6 General management

Management of patients with renal disease is multi-faceted (27). Treatments include symptomatic therapies such as diuretics; dietary interventions such as restricting sodium intake; management of secondary complications such as anaemia with erythropoeisis stimulating agents; treatment of secondary hyperparathyroidism; management of cardiovascular risk with lifestyle advice, anti-platelet therapy and statin treatment and treatments with the aim of retarding renal progression.

Current drug treatments employed in the management of CKD with the aim of retarding progression have one of three approaches – blood pressure reduction, inhibition of the renin-angiotensin-aldosterone system (RAAS) or immunosuppression. Immunosuppression is utilised in a targeted fashion in conditions thought to be primarily immune-mediated such as vasculitis. Secondly, antihypertensive agents are utilised with a target blood pressure of 140/90mmHg, falling to 130/80mmHg in the presence of more than 1g/day of proteinuria(27).

1.2.7 RAAS inhibition

Inhibition of the renin-angiotensin-aldosterone system with inhibitors of the angiotensin converting enzyme (ACEi) or blockers of the angiotensin II type 1 receptor (ARB) has revolutionised treatment of CKD(28). Beneficial effects on blood pressure do not adequately explain the significant benefits seen with these drugs in terms of reduction of proteinuria and retardation of progression of kidney disease. (29) The benefits of RAAS blockade are most marked in the context of dietary sodium restriction, since relative volume depletion results in greater angiotensin II dependence of the glomerular microcirculation (30). Other mechanisms of benefit include reduction of inflammation and fibrosis via alteration of podocyte behaviour and reduction in TGFβ1(31).

These agents are primarily indicated in patients with proteinuric renal disease either diabetic in origin (RENAAL study – losartan in type 2 DM)(19) (IDNT study – Irbesartan in diabetic nephropathy)(32) or non-diabetic (REIN study – ramipril)(33;34). There has however been a consistent failure to demonstrate a reduction in cardiovascular risk with these agents, despite limiting renal progression, suggesting alternative mechanisms of increasing cardiovascular risk are not being addressed via this therapeutic approach, leading to the pursuit of novel factors, such as mineralocorticoid blockade.

1.2.8 Mineralocorticoid receptor antagonism

This will be discussed in-depth in section 4.3.

1.2.9 Diabetic nephropathy

Diabetic nephropathy, progressive proteinuric kidney disease in the context of type 1 or type 2 diabetes, is characterised on light microscopy (Figure 1-2) by nodular glomerulosclerosis comprising mesangial expansion, nodule formation, mesangial hypercellularity and thickening of the glomerular basement membrane, which stains for IgG. (35) Initial pathological changes include a reduction in podocyte number per glomerulus, which is predictive of subsequent proteinuria (36), however these changes occur prior to the clinical phase of the disease and there are no therapies or investigations which are targeted at this.

Known risk factors for developing diabetic nephropathy include hypertension, poor glycaemic control, poor lipid control and smoking (37). UKPDS, a randomised trial of glycaemic therapy in type 2 diabetes, demonstrated the importance of optimising glycaemic control and managing hypertension in delaying onset of diabetic complications, including nephropathy(38). There is however evidence that there is genetic clustering of diabetic nephropathy in families(39), supporting a multi-factorial basis for the disease. Diabetic nephropathy is one of the commonest causes of end stage renal disease in Scotland and the incidence is increasing (40).

1.2.10 IgA nephropathy

IgA nephropathy is a primary glomerular disease whereby there is expansion of the mesangium (Figure 1-2) which stains positive for IgA antibody on immunofluorescence. It is associated with microscopic haematuria and proteinuria and has a variable progression rate, largely determined by blood pressure and levels of proteinuria(24). It is more common in men and studies suggest geographical variation in incidence, although this may simply reflect biopsy policy(41). It is the commonest primary glomerulopathy in Scotland(41).

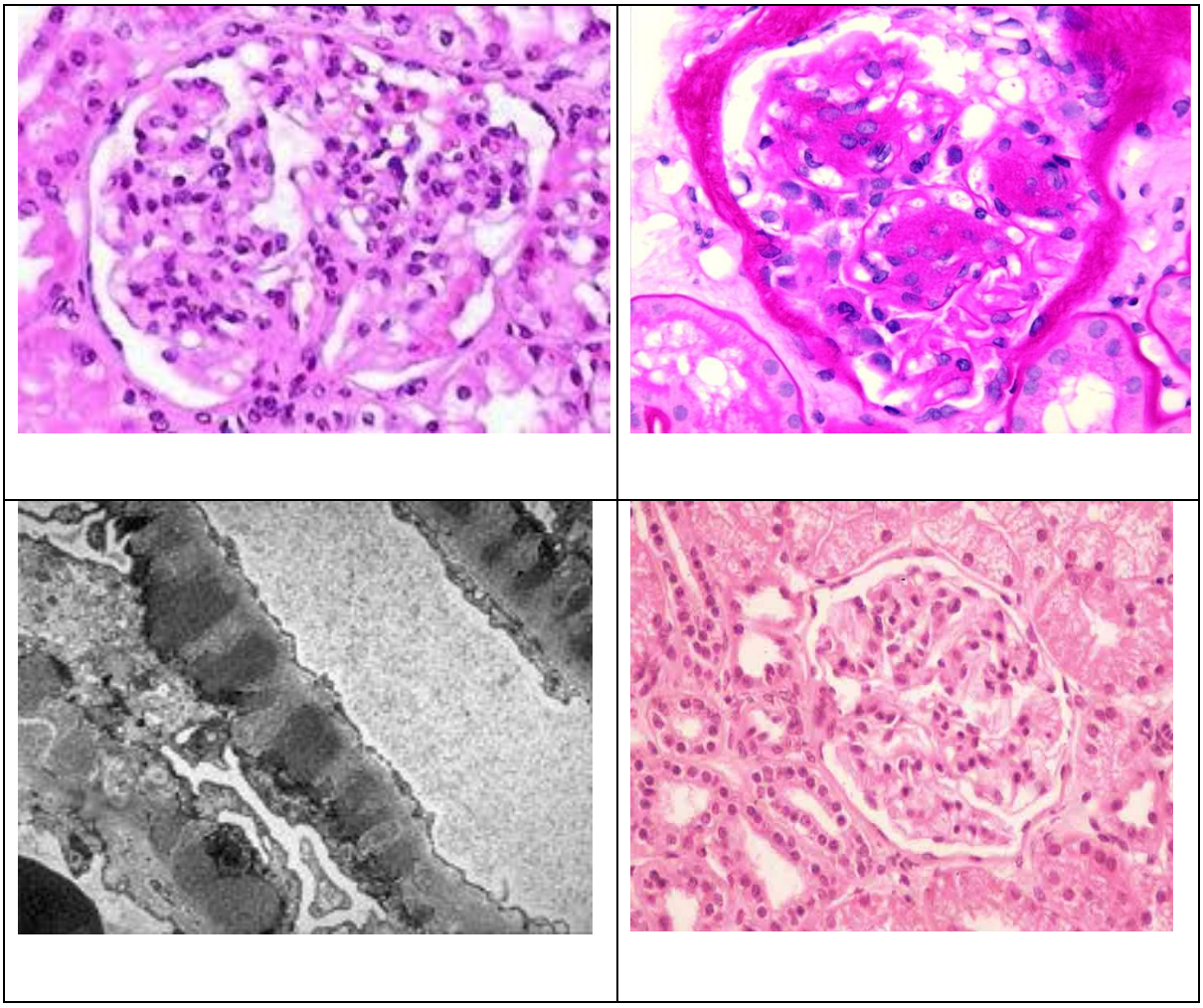


Figure 1-2: Renal biopsy specimens.

Upper left panel demonstrates IgA nephropathy with mesangial expansion and hypercellularity. Upper right panel demonstrates diabetic nephropathy with nodular glomerulosclerosis. Lower left panel shows electron microscopy of a glomerulus with membranous nephropathy and subendothelial immune complex deposition. Lower right panel, normal glomerulus seen under light microscopy.

1.2.11 Membranous nephropathy

Membranous nephropathy is the second most common primary glomerulopathy diagnosed on native renal biopsy and the commonest primary cause of nephrotic syndrome in Scotland(41;42). It is a condition characterised by subendothelial immune complex deposition resulting in significant levels of proteinuria. Recent evidence implicates the phospholipase A2 receptor in the disease pathogenesis(43). Membranous nephropathy can be seen secondary to recognised conditions but after excluding these, the majority of patients are found to have idiopathic disease.

1.3 Sodium

1.3.1 Normal homeostatic control of sodium excretion

Sodium is freely filtered at the glomerulus, with filtration depending upon three main factors. Firstly, the balance of hydraulic and oncotic pressures, secondly the ultrafiltration coefficient (determined by glomerular permeability and total filtration area of glomerular capillaries) and lastly the rate of plasma flow through the glomeruli (myogenic autoregulation).

The majority of sodium (70%) is reabsorbed in the proximal tubule via co-transport with organic molecules (urea, glucose, amino acids) and via the sodium-hydrogen ion exchanger. Active reabsorption occurs in the thick ascending limb via the NKCC2 channel (dysfunctional in Bartter's syndrome) where around 20% is reabsorbed. Five percent is reabsorbed in the proximal part of the distal convoluted tubule via the NCC channel (dysfunctional in Gitelman's syndrome) and fine tuning occurs in the collecting ducts via the epithelial sodium channel (ENaC) (2-4%).

Tubuloglomerular feedback is the mechanism whereby increased sodium delivery to the macula densa causes a reduction in renin secretion and AgII formation, with an increase in afferent arteriolar resistance and reduction in GFR. Changes in arteriolar tone are also mediated by vasodilatory factors (e.g. prostaglandins, atrial natriuretic peptide, nitric oxide) and vasoconstrictor factors (adenosine, ATP, vasopressin, angiotensin II, endothelins and adrenergic factors). Tubular sodium reabsorption is more important than GFR in regulating amount of sodium reabsorbed from the nephron, with angiotensin II enhancing proximal tubular sodium reabsorption and aldosterone promoting distal tubular

reabsorption(44). When patients are in steady state, urinary sodium excretion reflects dietary sodium intake.

1.3.2 Evidence for the role of sodium in blood pressure and kidney disease

1.3.2.1 Pressure natriuresis curve

Sodium has a key role in maintaining blood pressure. This is demonstrated by the pressure-natriuresis relationship, described by Guyton. Equilibrium pressure is the blood pressure obtained when the urinary sodium intake equals urinary sodium output (Figure 1-3). If the patient's blood pressure exceeds the equilibrium pressure, urinary sodium excretion is enhanced above sodium intake thus normalising blood pressure. This relationship is altered in renal failure, where the slope of the renal output curve is steeper due to the reduced renal mass, meaning that hypertension in these patients is traditionally salt sensitive i.e. blood pressure responds more closely to dietary sodium intake.

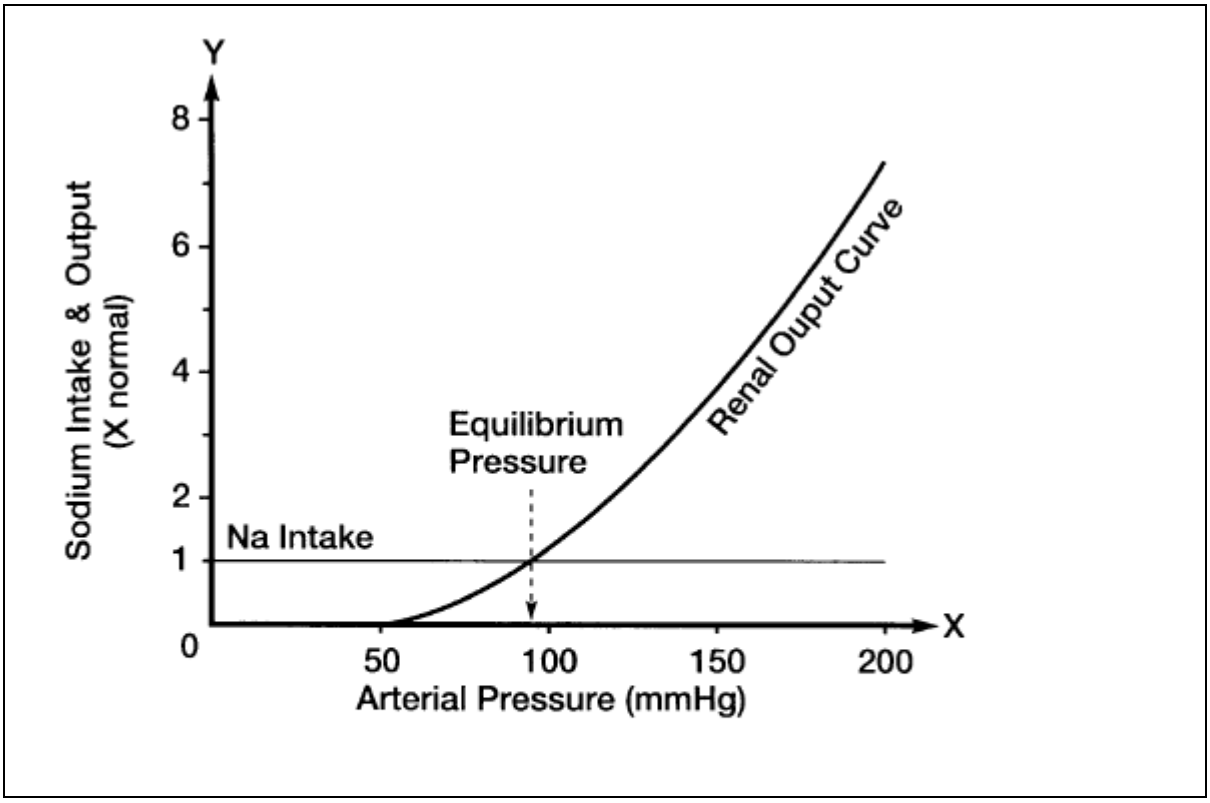


Figure 1-3: The pressure-natriuresis curve.

Between the mean arterial pressure on the X axis and the urinary sodium excretion rate on the Y axis, there is an approximately linear relationship (the renal output curve). When sodium intake is plotted, the blood pressure value at the intercept of the two lines is the equilibrium pressure at which the amount of sodium excreted in the urine becomes equal to the sodium intake, resulting in net sodium balance(45).

1.3.3 Measurement of total body sodium

Over time various measures have been used to assess sodium intake and total body sodium content. Urinary sodium excretion accurately reflects dietary sodium intake. Sodium is the predominant extracellular cation and 90% of body sodium is in this compartment. Measurement of total body sodium is difficult but a number of physiological studies were performed 30-40 years ago using a radioisotope method described by Davies et al to look at exchangeable body sodium utilising the ^{24}Na ($T_{1/2}$ 15h) radioisotope(46). By injecting a known quantity of isotope and then sampling the amount of isotope in the blood at 22h, adjusting for the amount excreted in the urine, exchangeable sodium can be calculated.

NaE = (^{24}Na injected - ^{24}Na excreted) / (serum ^{24}Na / serum sodium)

Muscle, skin, kidney and liver equilibriate with the isotope within an hour. Bone, brain and CSF take up to 20h to equilibriate and it is estimated that only around 40% of bone sodium equilibriates(47), therefore this method only estimates the amount of metabolically active sodium present.

Newer data, predominantly from Titze et al(48), supports a role for the non-osmotic storage of sodium in the skin and lymphatic system, via glycosaminoglycan binding. These data have come predominantly from animal studies but also from studying humans. The use of MR-spectroscopy to assess body sodium using ^{23}Na is non-invasive and attractive. Titze has validated this method in amputee muscle specimens and it is likely to be increasingly utilised, although still as a research tool(49).

1.3.4 Renin, angiotensin and exchangeable sodium in CKD

Historical studies demonstrate altered relationships between sodium, renin and blood pressure. In normal patients, a highly significant inverse relationship exists between exchangeable sodium and plasma renin concentration – a higher sodium is associated with a lower PRC. In patients with EH (50;51) a relationship between BP and level of exchangeable or plasma sodium was not consistently demonstrated. Furthermore, no relationship was consistently demonstrated between BP and renin or angiotensin levels in patients with EH.

In uraemia, total body sodium has been shown to be increased(52). Despite the presence of sodium retention and increased exchangeable sodium, plasma renin levels and angiotensin

II have been shown to be inappropriately high and renin failed to suppress normally with sodium retention in patients with uraemia (53).

1.3.5 Population blood pressure / sodium studies

Previous population studies have established dietary sodium intake to be a key mediator of blood pressure(54), with sodium reduction strategies demonstrated to result in a reduction in systolic blood pressure of 3 to 5 mmHg(55;56). Subsequent to this, long-term follow-up of these cohorts has demonstrated a 25% cardiovascular risk reduction in patients receiving a low sodium diet during preceding trial conditions(57). Further population extrapolations have suggested that salt-related blood pressure elevation accounts for 14% of strokes and 9% of myocardial infarctions(58). Damaging effects of dietary sodium on end-organs have also been shown in patients with hypertension, including increasing left ventricular mass and albuminuria (59) (60;61).

In patients with non-diabetic nephropathy, reduction in dietary sodium intake (mean urinary sodium excretion 106mmol/d versus 184 mmol/d) in addition to ACE inhibition reduced proteinuria and blood pressure more effectively than the combination of ACEi and ARB (62). As previously noted, inhibition of the RAAS is more effective in the presence of sodium reduction.

1.3.6 Sodium and CKD progression

Sodium intake is widely believed to influence progression of CKD, independent of effects on blood pressure(63). Certainly, a dietary approach to limiting progression would be attractive. There is experimental evidence to suggest a direct pathogenic role in renal failure(64). Clinical outcome data from cohorts of patients with CKD are sparse. A small cohort study from Italy(65) addressed the impact of sodium intake on progression of renal disease. They found patients with low urinary sodium excretion to have a lower creatinine clearance at baseline, but a slower loss in renal function. In patients with proteinuric nephropathy and stable CKD3, a low sodium diet resulted in a reduction in proteinuria over a 6 week period, independent of blood pressure which also reduced significantly. Aldosterone and renin levels increased during salt restriction(66).

The potential pathogenic mechanisms explaining sodium mediated vascular and renal damage are multiple. Haemodynamic effects mediated via volume retention, resulting in increased intraglomerular pressure and increased glomerular filtration are plausible; non-

haemodynamic factors such as oxidative stress via superoxide production(67) and inflammatory mediators have been widely studied in rat models (68) (69;70).

Increased expression of the pro-fibrogenic molecule TGF-β1 was seen in Sprague-Dawley rats treated for 4 weeks with a high salt diet (8.0% NaCl) compared with those treated with a low salt diet (0.3% NaCl)(68), with similar findings in aortic rings subjected to similar conditions (71;72). Modulating these effects is eNOS, with a parallel increased expression by the endothelium, which inhibits TGF-β1 expression (63). Normotensive and hypertensive rats fed a high salt diet developed vascular, glomerular and interstitial fibrosis(73). These studies suggest a directly nephrotoxic effect of dietary sodium.

In the rat anti-Thy 1 model of glomerulonephritis, sodium retention was seen, with reduced urinary sodium excretion mediated via a reduction of the sodium hydrogen exchanger type 3 (NHE3) in the proximal tubule and thick ascending limb of the loop of Henle and increased epithelial sodium channel (ENaC) expression in the collecting duct(74).

1.4 Aldosterone and other adrenal steroids – Humans

1.4.1 Aldosterone production

Aldosterone is produced in the zona glomerulosa of the adrenal cortex. It is produced from cholesterol which is translocated to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR), which is the rate limiting step in steroid hormone production. There then follows a series of enzymatic reactions (Figure 1-6) performed by dehydrogenases and cytochrome p450 oxidases. The final steps of converting deoxycorticosterone to aldosterone are performed by aldosterone synthase, which is encoded by the CYP11B2 gene. This enzyme is responsible for 11β-hydroxylation, 18-hydroxylation and 18-methyloxidation producing corticosterone, 18-hydroxycorticosterone and finally aldosterone.

There has been historical interest in extra-adrenal production of aldosterone in the central nervous system, heart and kidney but recent evidence suggests this is unlikely to occur and if it does may have little physiological relevance (75).

1.4.2 Physiological effects of aldosterone

Aldosterone is synthesised by the zona glomerulosa in response to a number of trophins – primarily angiotensin II, increased extracellular potassium concentration and adreno-corticotrophic hormone (76). Intracellular binding of aldosterone (Aldo) to the mineralocorticoid receptor (MR) in the distal tubular epithelium of the kidney results in translocation of the hormone:receptor complex to the nucleus and transcription of aldosterone inducible proteins e.g. serine threonine kinase (SGK1)(77). These in turn result in synthesis and translocation of the epithelial sodium channel (ENaC), promoting sodium reabsorption, and activation of the renal outer medullary potassium (ROMK) channels, extruding potassium into the lumen to maintain electrochemical neutrality. Sodium retention promotes water reabsorption and an increase in blood pressure(78).

1.4.3 Genetic control of aldosterone production

Aldo production has a heritable component, commonly reported to represent about 30% of variability. The C-344T polymorphism in the aldosterone synthase gene (CYP11B2)(79) is the most widely studied, with the T allele being associated with increased plasma Aldo and THAldo excretion rates. A further polymorphic variation, the intron 2 conversion, where intron 2 of the CYP11B2 gene is replaced with the corresponding region of CYP11B1, is in tight linkage disequilibrium with C-344T (Figure 1-4). The C-344T and intron 2 conversion are more frequent in patients with essential hypertension(80). The fundamental effects of these polymorphisms remain incompletely understood. This is explored by Connell et al(81).

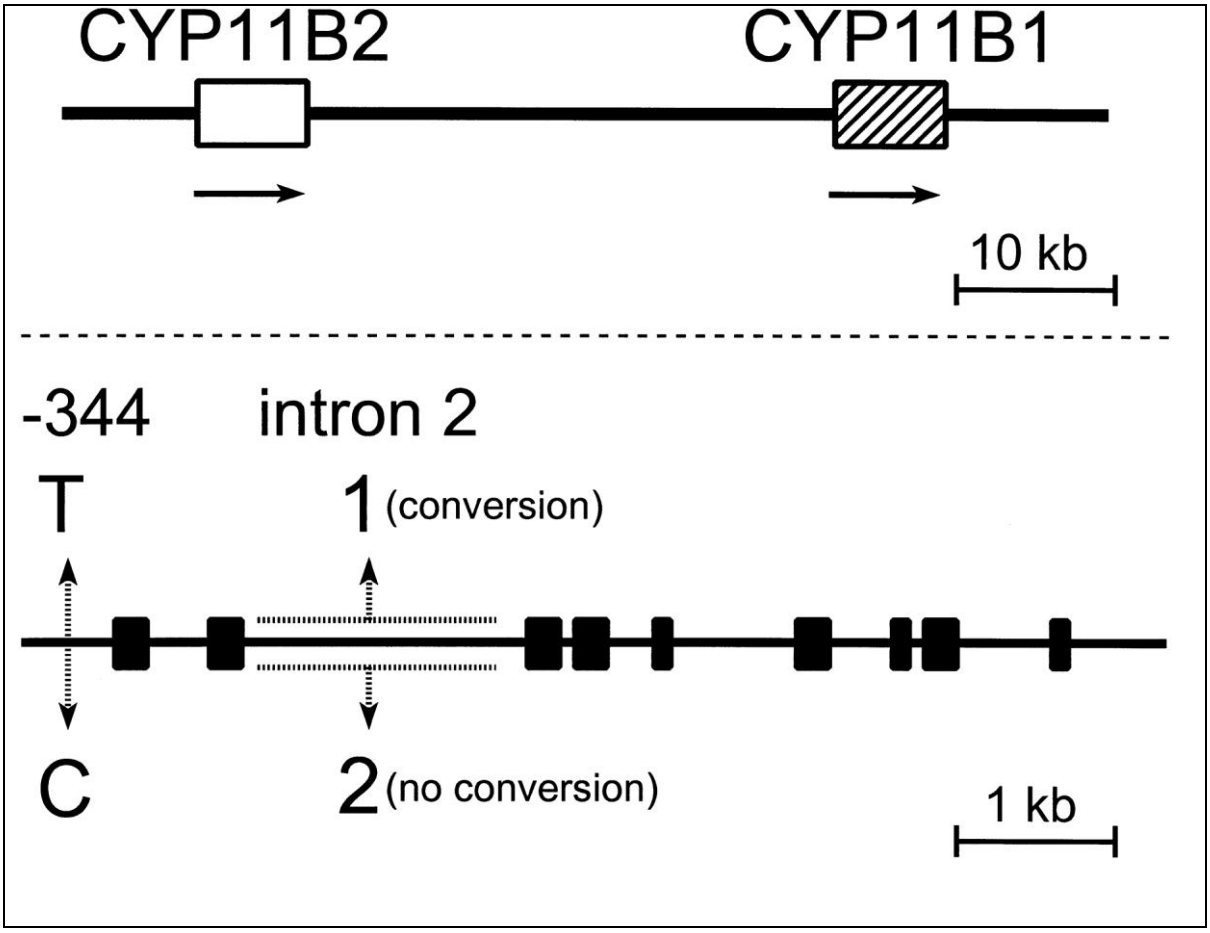


Figure 1-4: The CYP11B2 and CYP11B1 gene orientation (upper panel) and common polymorphisms of the CYP11B1 gene (lower panel).
Reproduced from Kupari et al(82).

1.4.4 Non-traditional role of aldosterone

Recent findings have widened the understood effects of Aldo to include so called non-genomic effects, which occur in response to Aldo but are too rapid to be accounted for by gene transcription, such as alterations in heart rate or vasoconstriction in response to blood loss or postural change (83).

In classic aldosterone target tissues, aldosterone has non-genomic effects where binding to the MR is followed by a rapid increase in intracellular calcium, followed by activation of the sodium-hydrogen ion exchanger, alkalinisation of the cell, extrusion of potassium and reabsorption of sodium. This process relies on protein kinase C, MAP kinases ERK 1/2 and epidermal growth factor(84). Inhibition of ERK 1/2 phosphorylation prevents the rise in intracellular calcium and activation of NHE(85). It is generally acceped that non-genomic effects of aldosterone often involve the MR and support genomic effects.

Similarly, aldosterone synthase, the main regulatory enzyme of aldosterone synthesis, has been located in tissues out with the adrenal cortex, particularly the vascular endothelium, brain and renal cortex, with synthesis occurring mainly in response to tissue damage(86). The MR additionally has been localized to the preglomerular vasculature, mesangial cells, fibroblasts as well as the distal tubular cells of the nephron(87). Therefore the roles of Aldo and the MR are likely to be more far reaching than initially understood.

1.4.5 Deoxycorticosterone

Deoxycorticosterone (DOC) is the Aldo precursor and substrate for aldosterone synthase. It is produced predominantly by the zona fasciculata of the adrenal gland, but only DOC produced in the zona glomerulosa can be used for aldosterone production. It is also formed extra-adrenally by 21-hydroxylation of plasma-borne progesterone(88), a mechanism which is not subject to negative feedback regulation. It is a weak mineralocorticoid which binds the MR with equal affinity to aldosterone but has a twentieth of the power of Aldo in terms of retaining sodium (77). Elevated levels of DOC can however be pathogenic(89;90), for example in rare patients with congenital adrenal hyperplasia and 11-βhydroxylase deficiency, where the DOC measured is predominantly under ACTH control and produced in the zona fasciculata. In these patients effects of mineralocorticoid excess (hypertension, sodium retention and volume expansion) are seen. Recent studies have also identified a DOC specific enzyme (AKR1C3) which converts DOC to an inactive metabolite (20α – hydroxyl-DOC) to allow MR binding to be restricted to aldosterone in the kidney and

colon(91). It has been postulated that variations in the expression of this enzyme may account for differing responses to DOC.

1.4.6 Adrenocorticotrophic hormone

The corticotrophin releasing hormone (CRH) – adrenocorticotrophic hormone (ACTH) axis has been studied in patients with CKD, with conflicting findings, largely due to problems with cortisol assays in renal failure(92). The most recent studies showed that baseline cortisol levels did not differ in patients requiring RRT compared with controls but there was a trend towards higher ACTH levels. ACTH responded normally to CRH but the cortisol response was blunted. The adrenals responded normally to synthetic ACTH (synacthen) stimulation(93;94). This suggests that the adrenal cortex responds near normally to ACTH stimulation.

1.4.7 Cortisol

Cortisol is the major human glucocorticoid. The production of cortisol by the zona fasciculata of the adrenal gland is largely controlled by ACTH which binds the promoter region of the CYP11B1 gene, altering production of the 11β-hydroxylase enzyme, catalysing production of cortisol from 11-deoxycortisol (Figure 1-6). The CYP11B1 and CYP11B2 genes are highly homologous, although the promoter regions differ(75). The major roles of cortisol are maintaining blood sugar levels, regulating the immune system and maintaining blood pressure during physiological stress. Cortisol and aldosterone have equal affinity for the mineralocorticoid receptor but specificity of the MR in the renal tubules is maintained by the 11β-HSD2 enzyme which converts cortisol to inactive cortisone.

1.4.8 11-β hydroxysteroid dehydrogenase

The 11-β hydroxysteroid dehydrogenase enzyme occurs in two main isoforms – type 1 and type 2. Type 1 converts inactive cortisone to active cortisol in the liver and adipose tissue and is assessed using the ratio of THF (tetrahydrocortisone) to THE (tetrahydrocortisol). It is inhibited by carbenoxolone. Type 2 is the enzyme predominantly found in the renal collecting tubules and converts active cortisol to inactive cortisone, protecting the aldosterone selectivity of the MR (Figure 1-5). The efficiency of this enzyme is assessed by measuring the ratio of free cortisone (F) to free cortisol (E). The ratio should measure between 2 and 3.

Studies have assessed activity of the 11-beta hydroxysteroid dehydrogenase (11-βHSD) enzyme in patients with CKD. In patients on HD, plasma cortisol:cortisone (F:E) and THF:THE ratios were increased, compared with controls, suggesting impaired enzyme activity(95). Similarly, in nephrotic patients the plasma THF:THE ratio was found to be higher than in controls(96). The enzyme of predominant interest in terms of CKD is the 11βHSD-2 isoform and the urinary F:E ratio is a more accurate measurement of this than the THF:THE ratio, which predominantly measures 11BHSD-1 activity(97). When the urinary F:E ratio was assessed in 8 nephrotic patients compared with 8 healthy controls there was no evidence of impaired enzyme activity(98). Quinkler et al however found that the urinary free F:E ratio correlated with level of renal function in patients with CKD(99). Sufficient ambiguity therefore exists relating to the function of this enzyme in the context of loss of renal function.

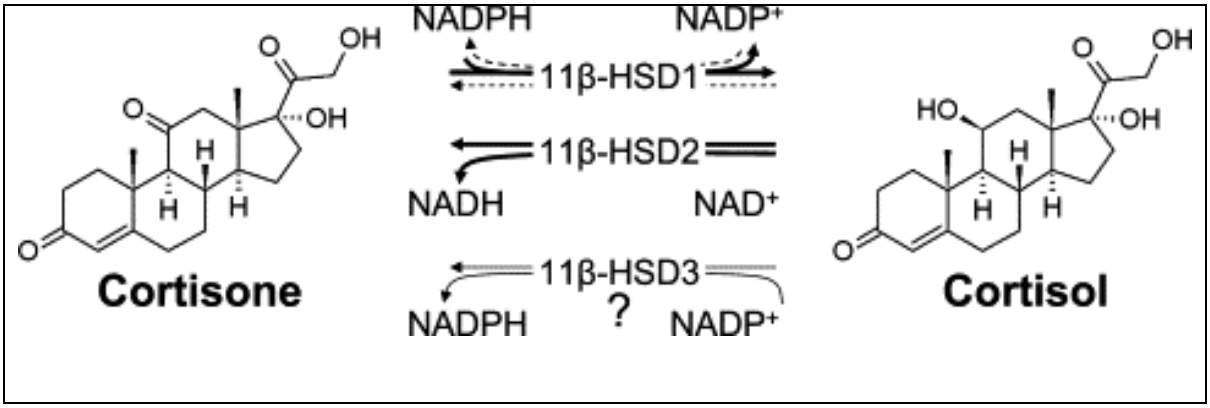


Figure 1-5: Diagramatic illustration of the opposite roles of 11-beta hydroxysteroid dehydrogenase isoforms.
Reproduced from Odermatt(100).

1.4.9 Metabolism and measurement of adrenal steroids

Metabolism of Aldo is predominantly hepatic, with 35-40% of Aldo in the urine being tetrahydroaldosterone (3-α, 5-β -tetrahydroaldosterone) (THAldo), due to metabolism with 3β-hydroxysteroid dehydrogenase and 3-alpha-hydroxysteroid dehydrogenase, followed by conjugation to glucuronic acid. 0.5% of secreted Aldo is free (unconjugated) aldosterone. Aldosterone-18-glucuronide is produced by direct conjugation of Aldo, largely in the kidney, accounting for 5-10% of Aldo metabolites in urine(101).

Measurement of urinary metabolites of Aldo over 24h has been shown to provide a more accurate representation of Aldo production, as it averages out short term fluctuations associated with timing and posture(101). Urinary metabolites of cortisol (THE, THF and aTHF) are recorded as a measure of cortisol production. Urinary free cortisol (F) and cortisone (E) are considered and the ratio between them as a measure of 11β-HSD2 enzyme activity. The ratio of 11-deoxycortisol (S) or it's principal metabolite (THS) to cortisol is another index of potential mineralocorticoid activity.

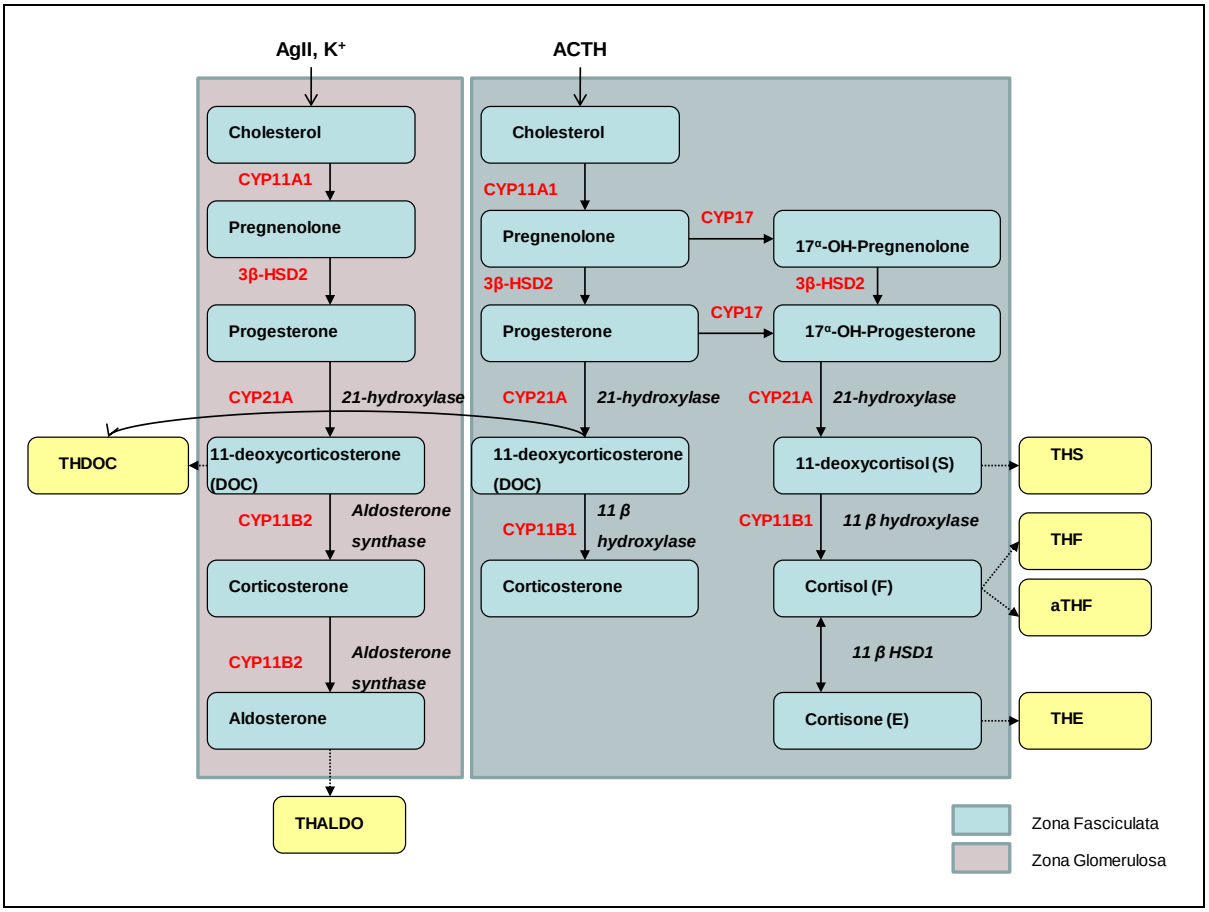


Figure 1-6: Adrenal steroid synthesis and pathway with dominant urinary metabolites in yellow, enzymes in italics and genes in red.

Metabolites: THDOC = tetrahydrodeoxycorticosterone; THALDO = tetrahydroaldosterone; THS = Tetrahydrodeoxycortisol; THF = Tetrahydrocortisol; aTHF = Allotetrahydrocortisol; THE = Tetrahydrocortisone. AgII = Angiotensin II; K = Potassium; ACTH = adrenocorticotrophic hormone.

1.4.10 Drug and obesity effects on aldosterone levels

The levels of aldosterone and renin are directly affected by drugs used to treat hypertension (Table 1-1). This should be borne in mind when interpreting results. Plasma aldosterone concentration (PAC) levels have also been shown to be increased in patients who are obese(102), with oxidized fatty acids being thought to stimulate aldosteronogenesis(103). Levels are also higher in patients of black origin(104).

Multiple questions therefore remain relating to the control of mineralocorticoid synthesis in the uraemic milieu. Data which inform our understanding of this situation are largely historical and relate to patients with significantly impaired renal function. Similarly, current clinical practice is greatly influenced by the prescription of medications known to interfere with normal levels of mineralocorticoids, and how to understand and interpret this in patients with CKD has not been studied.

Medication class	Aldosterone	Renin
ACE-inhibitor	↓	↑
ARB	↓	↑
Beta-blocker	↓	↑↑
Potassium sparing diuretic	↑	↑
Potassium wasting diuretic	↑	↑
NSAID	↓	↓
Dihydropyridine calcium channel blocker	→	↑

Table 1-1: Effects of medication on measurement of plasma aldosterone and renin(105).

1.4.11 Steroid related genetic effects causing hypertension

A number of rare genetic mutations have been associated with steroid related hypertension. These are detailed in Table 1-2.

Condition	Mutation	Effect	Treatment
Liddle's syndrome	Autosomal dominant ENaC subunit mutation	Impaired ENaC degradation with enhanced Na reabsorption, hypertension, low PRC, low PAC, hypokalaemia, metabolic alkalosis	Amiloride
Glucocorticoid-remediable aldosteronism	Chimeric gene formed from CYP11B1 and CYP11B2 – autosomal dominant	Hyperaldosteronism secondary to ACTH sensitive aldosterone production in the zona fasciculata	Dexamethasone
Syndrome of apparent mineralocorticoid excess (SAME)	11α-hydroxysteroid dehydrogenase type 2 mutation	Cortisol:MR binding and clinical mineralocorticoid excess	Carbenoxolone and liquorice
Congenital adrenal hyperplasia	11β-hydroxylase deficiency due to CYP11B1 mutation	Impaired cortisol production, androgen excess, ACTH and DOC excess	Replacement
Congenital adrenal hyperplasia	17α-hydroxylase deficiency - CYP17 mutation	Hyporeninaemia, hypoaldosteronism, hypokalaemia, hypocortisolaemia, absent secondary sexual characteristics. DOC excess.	Replacement
Gordon's syndrome	Autosomal dominant WNK gene mutation	Pseudohypoaldosteronism type 2 – hypertension, hyperkalaemia and acidosis. Increased NCC activity	Thiazide diuretic

Table 1-2: Steroid biosynthesis defects associated with hypertension.

1.5 Role of aldosterone in hypertension and kidney disease

1.5.1 Aldosterone and hypertension

Hypertension is a key determinant of cardiovascular risk and progression of renal dysfunction(87;106;107). Aldo contributes to the development of hypertension in the general population. In the Framingham offspring study(108), variation in Aldo levels across the normal physiological range accounted for change in blood pressure over a five year period and development of hypertension. Additionally, in up to 15% of hypertensive patients, excessive Aldo levels, in relation to renin, defined by a raised ratio of aldosterone to renin (ARR) is described(109). Evidence of excessive Aldo production is found in up to 20% of patients with resistant hypertension(110). However, although high blood pressure is very common in patients with CKD, the role of Aldo in these patients in raising blood pressure and contributing to progression of renal dysfunction is unknown.

1.5.2 Risks associated with hyperaldosteronism

Patients with primary aldosteronism have significantly worse cardiovascular outcomes than matched essential hypertensive subjects(111). Additionally, Aldo levels are directly correlated with adverse outcomes after myocardial infarction(112). There is also evidence that higher Aldo levels within the normal range, as well as proven hyperaldosteronism, in the context of hypertension, are associated with microalbuminuria(113). Whether this reflects endothelial dysfunction secondary to oxidative stress, or a consequence of hyperfiltration due to direct Aldo effects on the pre and post glomerular arterioles is controversial(114). Patients with hypertension secondary to hyperaldosteronism initially demonstrate a sharp decline in glomerular filtration rate with treatment, which stabilises after 6 months and thereafter renal decline does not differ between patients with hyperaldosteronism and essential hypertensives(114;115). The initial sharp reduction is thought to represent a reversal of hyperfiltration.

Isolated hyperaldosteronism does not appear to be pathogenic in itself, in the context of a low dietary sodium intake and high potassium intake. This is exemplified by studies on Yanomamo Indians, where their primitive lifestyle and rudimentary diet results in high aldosterone production but low blood pressure and minimal cardiovascular disease(116).

1.5.3 Aldosterone and the kidney – adverse effects in humans

One recent study, in hypertensive humans with normal kidney function, demonstrated a relationship between Aldo, sodium excretion and urinary protein excretion, with proteinuria being greatest in subjects with both high Aldo levels and high urinary sodium excretion(117). An analysis of the Framingham cohort(118) demonstrated an association between high urinary sodium and urinary protein excretion and high serum Aldo and protein excretion, but no interaction between the two. Bianchi demonstrated a relationship between plasma aldosterone concentration and proteinuria excretion(119). However, there are no studies in man that have examined the relationship between Aldo and sodium status and proteinuria in patients with CKD.

1.5.4 Mineralocorticoid levels and the adrenal axis in chronic kidney disease

Traditionally it has been accepted that the plasma Aldo concentration rises in renal failure. Findings from a small historical cohort showed that once creatinine clearance falls below 60ml/min(120) the aldosterone : renin ratio (ARR) rises (Figure 1-7). The main statistically significant increase in plasma Aldo concentration (PAC) was however seen in patients with a creatinine clearance between 3-10ml/min and the wide scatter at this level suggests the effect is not uniform.

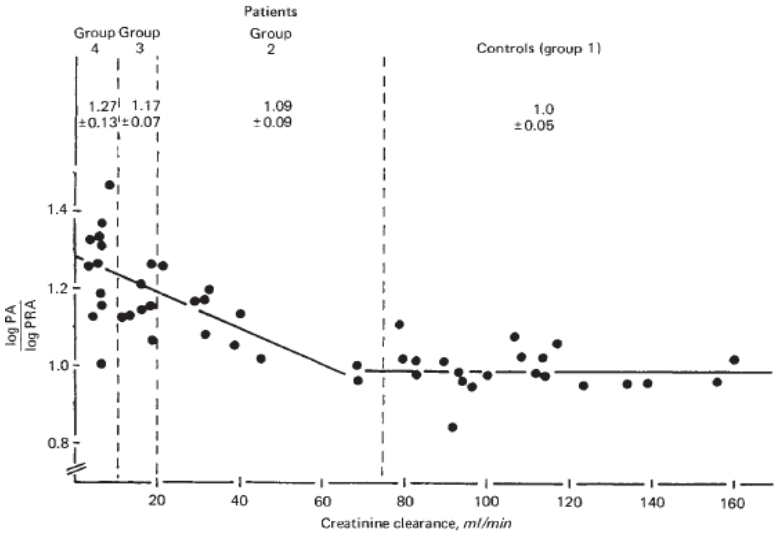


Fig. 1. *Relation between log* PALDO/*log PRA and creatinine clearance.* For the patient group, $r = 0.54$ ($P < 0.007$).

Figure 1-7 Relationship between the aldosterone:renin ratio and creatinine clearance(120).

Aldosterone : renin ratio derived from upright early morning samples in patients with CKD and controls.

Berl et al in 1978 assessed 8 patients with CrCl <15ml/min and found PAC to be above the reference range for the assay in 5 out of the 8. They also found that patients responded normally to alterations in posture and sodium restriction(121), suggesting the cortex responds appropriately. In patients on haemodialysis, one study showed 50% of patients had an elevated PAC but this was not associated with a worse mortality(122).

Previous studies predominantly utilise plasma measures, but a more recent study assessed plasma and urinary Aldo levels in patients with CKD 3 and low levels of proteinuria on ACEi / ARB therapy. They found PAC correlated negatively with eGFR but found no association between eGFR and THAldo excretion. As metabolism of Aldo is predominantly hepatic, with principal circulating metabolites being inactive, increased PAC or THALDO levels should not relate to reduced renal function.

1.5.5 Mineralocorticoid receptor blockade in humans – delaying progression of renal disease

Evidence that existing therapies delay progression or prevent cardiovascular complications in renal disease is limited. In patients with diabetic nephropathy and primary glomerulopathies, agents which block the RAAS reduce the rate of progression to end stage renal disease(19). However, there are patients in whom this treatment has no effect, or the effect of ACE inhibition or ARBs is not complete and patients continue to progress. The addition of one drug to the other has additional antiproteinuric effects; however whether the failure of monotherapy to be effective is due to insufficient drug dose, to non-angiotensin converting enzyme generation of angiotensin II or breakthrough of Aldo production is unclear (123). ‘Aldosterone breakthrough’, i.e. inappropriate elevation of serum Aldo levels in the context of blockade of the renin-angiotensin system, has been estimated to occur in up to 40% of patients and has been associated with more rapid decline in renal function in patients with nephropathy(124). This has contributed to interest in MR blockade as a potential target for inhibiting renal progression.

Mineralocorticoid receptor blockers (MRBs) include spironolactone and eplerenone. Spironolactone is a synthetic steroid which competitively inhibits intracellular MR:Aldo binding in the distal convoluted tubule. It also has anti-androgen activity by binding the androgen receptor, leading to gynaecomastia in men. Eplerenone is similar to spironolactone but is more selective and does not have the anti-androgen effects.

MRBs have been shown to significantly reduce mortality in patients with heart failure or post-myocardial infarction(125;126). In proteinuric CKD patients, there have been a number of studies demonstrating beneficial effects of MRBs both in terms of proteinuria reduction(127;128) and slowing of decline in GFR(119;129). However, the studies are small, diverse and have short follow-up. A recent Cochrane review supported the conclusion that MRBs contribute to the reduction in proteinuria in patients with CKD prescribed ACEi or ARB but are associated with an increase in hyperkalaemia(130). One study has examined the cardiovascular effects of spironolactone on LV mass in patients with CKD and demonstrated a significant reduction, independent of blood pressure(131). That cohort was studied further and shown to have improved LV systolic function and reduced pro-BNP levels(132). Carotid intimal media thickness(133) and aortic pulse wave velocity(131) have both also been shown to be favourably reduced in HD and CKD patients respectively with MR blockade.

Whilst these studies are intriguing, none provide definitive information on the role of Aldo, and on the dependency of an interaction with sodium status, in determining renal risk and cardiac and vascular function in CKD patients. Similarly, clinical studies have failed to demonstrate whether the beneficial effects of MR blockade relate to a generic effect, or specific inhibition of Aldo, the traditional MR ligand.

1.6 The relationship between aldosterone levels and sodium intake - in vivo studies

A number of historical studies underpin current physiological understanding of the relationship between sodium and aldosterone. The primary physiological role of Aldo is sodium reabsorption, thus maintaining intravascular volume and pressure. In the situation of a high sodium load, renin and Aldo production are suppressed(134). Similarly, Aldo is important for potassium extrusion and levels increase in a linear fashion with potassium loading(135). Sodium depletion results in a steepening of the aldosterone dose response curve to angiotensin II(136) – i.e. the adrenal cortex is more sensitive in sodium depletion. In renin-dependent hypertension, the AgII-Aldo response relationship is also steepened(50).

Similarly, angiotensin II levels increase with sodium deprivation(53) and in normotensive subjects, exchangeable sodium is inversely correlated with AgII levels and plasma renin concentration(137).

In the context of hyperaldosteronism, the usual feedback mechanisms are lost with the excess Aldo producing deleterious effects. The negative effects have been most comprehensively studied in animals.

1.6.1 Excess aldosterone and the heart - in vivo studies

Studies in animal models have shown that Aldo exerts major pro-inflammatory and fibrotic effects in the heart and kidney via the MCR, mainly seen in the context of a high sodium intake(87;138). In 1993 Brilla et al(139) demonstrated that the uninephrectomized rat, fed a high salt diet and infused with Aldo, developed cardiac perivascular and interstitial fibrosis. These effects were blocked with spironolactone but not captopril. The results were confirmed by Young et al (140). The cardiac fibrosis was demonstrated equally in both ventricles, suggesting a direct humoral effect, rather than being related to altered haemodynamics. Coadministering spironolactone(141) or canrenoate(142) blocked the hypertrophy and fibrosis, at doses which had minimal effects on blood pressure. The timescale for these effects to be seen was of the order of 4 weeks (143).

Similarly, in a rat model where the rats were treated with AgII, L-NAME (a NOS inhibitor) and saline, significant vascular damage, proteinuria and renal arteriopathy was seen at 14 days(144). These effects were not seen in rats treated with adrenalectomy or eplerenone, suggesting mineralocortioids had a central role in the process. The effect of eplerenone was more marked in the heart than the kidney and the protective effects of adrenalectomy were lost when Aldo was infused. Notably, the rats did not develop fibrosis, leading the authors to hypothesise that this occurs at a later stage as a consequence of the vasculopathy.

Extrapolating these findings to humans with long-term mineralocorticoid excess may be difficult, but it may be possible to compare the situation to acute myocardial infarction, where there is some evidence of AgII stimulated local Aldo synthesis in rats and inappropriate MCR activation, which is blocked by spironolactone, limiting cardiac remodelling(145). Recent evidence however questions the significance or indeed existence of cardiac aldosterone synthesis(75).

1.6.2 Excess aldosterone and the kidney - in vivo studies

In 1996, Hostetter demonstrated that in the 5/6 nephrectomy rat model, rats treated with an ACE inhibitor (enalapril) and angiotensin receptor blocker (losartan), infused with Aldo

and fed a normal salt diet developed hypertension and glomerulosclerosis(146). Rocha confirmed these findings using the stroke prone spontaneously hypertensive rat fed a high salt diet, finding that the rats developed severe glomerular injury with vascular fibrinoid necrosis and thrombotic microangiopathy, leading to renal fibrosis(147;148). Figure 1-8 illustrates some of these findings.

Uninephrectomised rats treated with Aldo and salt also produce an inflammatory response, whereby there is increased expression of a number of inflammatory molecules including ICAM-1(149), IL-1β, IL-6 and osteopontin(150); which in turn leads to hypertension and glomerulosclerosis. Adrenalectomised spontaneously hypertensive stroke prone (SHRSP) rats infused with Aldo produce profibrotic factors including TGF-B1 and PAI-1 resulting in renal injury. This pro-inflammatory effect was not seen in similar animals infused with angiotensin II(151). Aldo has also been shown to increase NADPH and oxidative stress in rats with Aldo/high salt induced renal injury(138).

It was previously thought that these proinflammatory and profibrotic effects were not seen in normal, normotensive rats. However it has been shown that adrenalectomised mice maintained on 0.9% saline and infused with Aldo exhibit renal collagen 1 accumulation prior to the development of hypertension(152), demonstrating that these changes can occur without prior renal injury or hypertension. The effect was not seen with corticosterone. Transgenic mice with hyperaldosteronism but normotension do not however develop cardiac hypertrophy or fibrosis(153). This is consistent with findings in Yanomamo Indians who have chronically elevated aldosterone levels secondary to a low sodium high potassium diet, but normotension and excellent vascular health(116).

Spironolactone has been shown to inhibit acute tubular necrosis in the ischaemia reperfusion model(154) and induce regression of existing glomerulosclerosis(155). Subsequently Aldo has also been shown to have a role in acute and chronic(156) calcineurin nephrotoxicity.

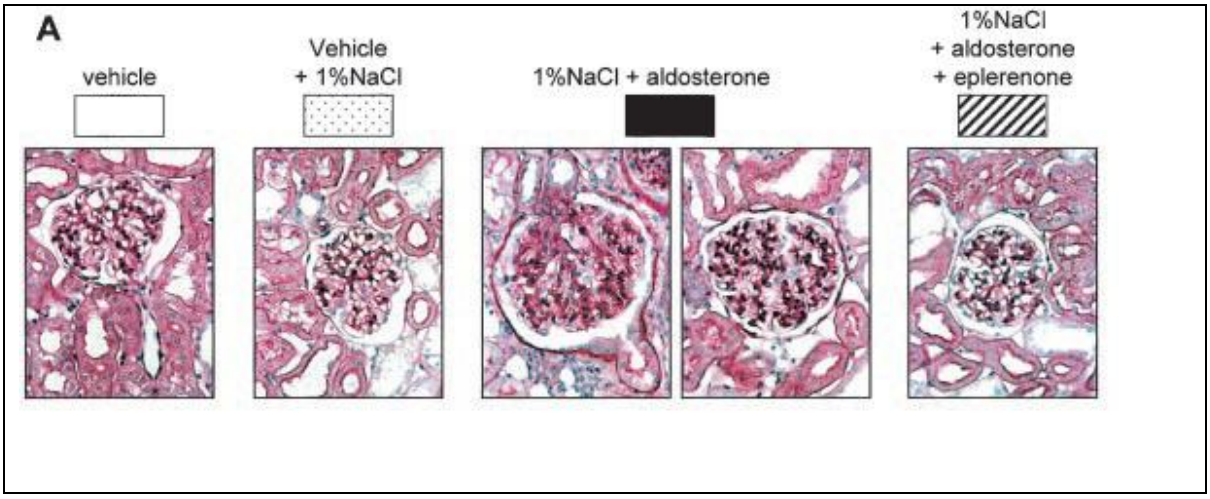


Figure 1-8: Aldo/1% NaCl-treated rats exhibit damaged glomeruli characterized by mesangial matrix expansion and cell proliferation, which is blocked by the addition of eplerenone (Reproduced from Nishiyama et al (138)).

1.6.3 The DOCA salt rat

Aldosterone is not the only mineralocorticoid demonstrated to have negative effects. The DOCA-salt rat is a model of hyperaldosteronism in humans, whereby administration of a high concentration of deoxycorticosterone acetate, a high salt diet and often uninephrectomy causes hypertension within 3 weeks(90), with DOCA treatment causing a 30-fold increase in sodium absorption(157) secondary to increased sodium channel activity. The initial sodium and fluid retention seen results in hypertension, with enhanced sympathetic activity and elevated levels of endothelin-1, increasing vascular resistance. In humans, deficiency of 11β-hydroxylase or 17α-hydroxylase results in elevated circulating DOC concentrations leading to mineralocorticoid hypertension in the majority of patients.

1.7 In vitro studies of renal fibrosis: inflammatory mediators, aldosterone and sodium

Renal fibrosis is the pathological hallmark of chronic kidney disease. Aldo has been shown to have significant pro-inflammatory and pro-fibrotic effects on cultures of renal, vascular and cardiac cells. No studies have looked directly at the combined effects of aldosterone and sodium on cell culture, out with the heart, despite evidence that the negative effects of mineralocorticoids are predominantly seen in a high sodium environment.

1.7.1 Transforming growth factor beta

TGFβ1 is a key mediator in the development of renal fibrosis. It has a number of physiological functions including regulating proliferation and differentiation of cells, tissue repair and angiogenesis(158), with effects depending on the target cell(159). TGF-β downregulates the matrix metalloproteinase collagenase, promotes fibroblast proliferation and upregulates collagen synthesis(160). Virtually every cell in the body produces TGFβ and has receptors for it. TGFβ1 is expressed in endothelial, haematopoietic and connective tissue cells, TGFβ2 in epithelial and neuronal cells and TGFβ3 in mesenchymal cells. In normal rat kidney TGFβ1 expression was localised to the afferent and efferent arterioles, particularly the cytoplasm of smooth muscle cells rather than the endothelium. TGFβ2 stained strongly in the basolateral aspect of distal tubules and weakly in the proximal tubules. TGFβ3 was strongly expressed in tubules, especially the brush border of proximal tubules of the other cortex of the kidney.(161)

TGF β1 impacts principally at tubules in the renal cortex and medulla and is an important candidate for Ang II mediated matrix synthesis(162). TGF β1 upregulates cellular volume responsive kinase, hSGK1 (serum glucocorticoid inducible kinase 1), which stimulates epithelial sodium channels. An increase in cellular sodium inflow promotes enhanced cell swelling, proteolysis inhibition, increased protein synthesis and extracellular matrix expansion(162). Aldo stimulates TGFβ1 production in rat mesangial cells, an effect blocked by the addition of spironolactone(163). Aldo infusion in normal rats for 3 days increased urinary TGF-β excretion, with no effect on blood pressure or histology, an effect which was lost with the addition of spironolactone(164).

Plasma levels of TGFβ 2 and 3 are undetectable, suggesting their effects are paracrine or autocrine(165). A study on human proximal tubular cells, whereby TGFβ1 was effectively silenced using small interference RNA methodology(166), demonstrated that silencing TGFβ1 was associated with a 3-fold increase in TGFβ2 and 2-fold increase in TGFβ3 measured by PCR, suggesting that TGFβ1 endogenously inhibits TGFβ2 and 3. TGFβ2 and TGFβ3 have been shown to stimulate TGFβ1 production in rat mesangial cells. These different isoforms have not been closely studied in CKD.

1.7.2 GM-CSF, IL6, IL8

Granulocyte/macrophage colony-stimulating factor (GM-CSF) is a pro-inflammatory cytokine produced by stimulated cells in response to IL-1 or LPS. In vitro it acts as a mediator to stimulate cell proliferation and activation, commonly cells of macrophage lineage, increasing inflammation(167). GM-CSF deficient mice fail to develop crescentic glomerulonephritis(168), indicating that it has a role in the pathogenesis of renal disease. IL-6 is a pro-inflammatory cytokine produced by a variety of cells in response to IL-1(169) and which has been shown to increase the signalling response to TGF-beta(170). IL-8 is a proinflammatory cytokine secreted by a number of cells, typically monocytes and macrophages, which encourages cellular recruitment, amplifying the immune response (171).

1.7.3 Collagen

Collagens are the main component of connective tissue. They aggregate as fibrils made up of three alpha chains. Collagen 1 is the most abundant collagen and is the main component of fibrotic tissue. Collagen 2 forms hyaline cartilage, collagen 3 forms granulation tissue, before being replaced by collagen 1, and collagen 4 forms the glomerular basement

membrane. Accumulation of collagen, predominantly 1, 3 and 4 (26), is seen in interstitial fibrosis, the dominant histological feature of CKD.

1.7.4 Aldosterone mineralocorticoid receptor binding

The intracellular signalling pathways downstream of Aldo binding to the MR have been characterised, but the exact mechanisms by which the pro-inflammatory and pro-fibrotic effects are exerted are not clear, and the way in which sodium status influences the response to activation of the MR is not understood. A new protein kinase family has become the subject of much study, the with-no-K[Lys] protein kinases (WNK)(172); their role in controlling sodium channels is of interest in relation to mineralocorticoid binding.

1.7.5 Renal sodium reabsorption

Renal tubular sodium reabsorption is a major determinant of renal oxygen consumption(173) and sodium overload might cause hypoxia, development of reactive oxygen species, production of NF-κB, local production of Ang II and a resultant pro-inflammatory response(174).

Normal Sprague-Dawley rats were exposed to a range of sodium loads, without haemodynamic changes, and a corresponding increase in TGF β1, RANTES, NF- κB and Ang II expression was seen on renal histology(162). Intracellular dehydration also promotes NF- κB production and may be contributing to the effects seen. Similarly, studies of rat aortic endothelial cells demonstrated that cellular salt intake exerts a dose-dependent increase in endothelial production of TGF-B1 via shear force and MAPK activation(68;175;176).

Cultured cardiomyocytes treated with sodium (146 mmol/liter) shrink in size, an effect which was inhibited by Aldo, via activation of the NHE1 receptor. Treatment was accompanied by an increase in intracellular pH(177), cell hypertrophy, and BNP production.

1.7.6 Intra-renal RAAS

The kidney is known to be a site of local AgII production, which is thought to act in a paracrine or autocrine fashion. The AT1 receptor is the dominant AgII receptor, mediating the classic effects of AgII. It is widely expressed within the kidney vasculature, glomerulus

and tubule. The AT2 receptor is also found within the kidney although it's precise role is less clear and thought to be antagonistic to AT1(178). AgII acting via the AT1 receptor directly inhibits renin release from juxtaglomerular apparatus. Renin mRNA has however also been located in the collecting duct and the intra-renal RAAS is activated early in the course of diabetes.

Prorenin, the renin precursor, binds the prorenin receptor and becomes enzymatically active, possibly increasing local AgII production. In mesangial cells, prorenin receptor binding results in a profibrotic phenotype(179), but the overall pathological significance of prorenin remains to be elucidated. Detection of angiotensinogen in the urine is thought to be good evidence for the existence of an intrarenal RAAS because it is unlikely to be filtered because of its molecular weight. Levels have been found to be elevated in patients with chronic kidney disease and associated with an increased risk of renal progression(180).

1.7.7 In vitro effects of aldosterone

In cultured mesangial cells, Aldo promotes expression of CTGF, TGF-β, collagen IV and fibronectin(149) via activation of SGK-1. HKC cells (human kidney cortex cells) treated with Aldo were shown to increase gene and protein expression of collagen types III and IV, with no effect on collagen I and II, an effect inhibited by the addition of spironolactone or U0126, an ERK inhibitor(181). In rat renal fibroblasts, Aldo can stimulate gene expression and synthesis of collagen I, III and IV (182). Similarly, in inner medullary collecting duct cells, Aldo alone increased collagen expression two-fold whilst cortisol alone did not after 48h, an effect blocked by MR inhibition (152).

Aldo also induces collagen synthesis in cultured cardiac fibroblasts(183), an effect inhibited by spironolactone(184). Additionally, Angiotensin II has been shown to induce collagen 1 gene expression in mice aorta and renal cortex in vivo(185) as well as stimulating cell proliferation and matrix synthesis in renal fibroblasts(186).

Another mechanism by which Aldo promotes a pro-fibrotic phenotype is via epithelial-mesenchymal transition (EMT). EMT is the process by which tubular cells lose their epithelial phenotype and migrate to the interstitium, adopting a myofibroblast phenotype, determined in vitro by the loss of E-cadherin (an epithelial marker) and the expression of

α-SMA (a myofibroblast characteristic). Aldo has been shown to induce EMT in HKC cells(181) and HK-2 cells(187).

Mesangial cell (188), cortical collecting duct cell(189), vascular smooth muscle cell(190) and cardiac fibroblast(191) proliferation has been induced by Aldo, via ERK signalling and variably via MCR binding. Conversely, Aldo has also been shown to promote apoptosis in HK-2 proximal tubular cells with up to 30% of cells having apoptosed at 24h when treated with Aldo (10^{-6}M), an effect occurring through oxidative stress and mediated by the MR (192). Of note, this is a high dose. A similar effect was reported in podocytes(193).

1.8 Left ventricular mass in CKD

Left ventricular hypertrophy (LVH) is a predictor of cardiovascular and all cause mortality in patients with hypertension and patients with CKD (194;195). Reduction of LV mass has been associated with reduced mortality in a variety of groups at high cardiovascular (CV) risk (196). Patients with CKD have increased CV risk, above that of the general population, which increases as renal function declines (5). However, the mechanisms responsible for this increased risk remain poorly elucidated, with the majority of interest focussing on patients with end stage renal disease (ESRD).

1.8.1 Measurement

1.8.1.1 Echocardiography

Existing studies of LVH in renal disease have used echocardiography or ECG. The dependence of echocardiography on intravascular volume leads to an overestimation of LVMI in patients with advanced renal disease(197) and whilst ECG criteria can be applied, this is generally less reliable(198).

1.8.1.2 Cardiac magnetic resonance imaging

Magnetic resonance imaging (MRI) maps proton density (hydrogen nuclei) by utilising the proton spin in a high strength magnetic field, with the application of radiofrequency pulses.

Normally, protons spin randomly, but the application of a magnetic field will result in protons aligning to the Z-axis, either in a parallel or anti-parallel direction, where they will continue to precess at their own frequency (Larmor frequency). A small excess of protons is always in the low energy state, resulting in a net magnetisation vector. The application

of a radiofrequency pulse results in alignment of the phase of the spins and an increase in the number of protons in the high energy state, altering the net magnetisation vector (Figure 1-9).

Cessation of the signal results in protons re-emitting energy and reverting to the lower energy state. This releases a signal in the form of radio waves, which induces a voltage across the coil. Reverting to the lower energy state occurs in two distinct processes – longitudinal relaxation (T1) and transverse relaxation (T2) (Figure 1-10). Differing T1/T2 properties of different tissues results in different signal intensities at different times, allowing differentiation between tissues. Weighting of the image allows focus on different properties. In T1 weighted images fluid is black because fluid has a high T1, meaning that the application of a second radiofrequency pulse affects relatively few protons as they are still relaxing from the previous pulse, therefore there is little signal. In T2 weighted images fluid is white.

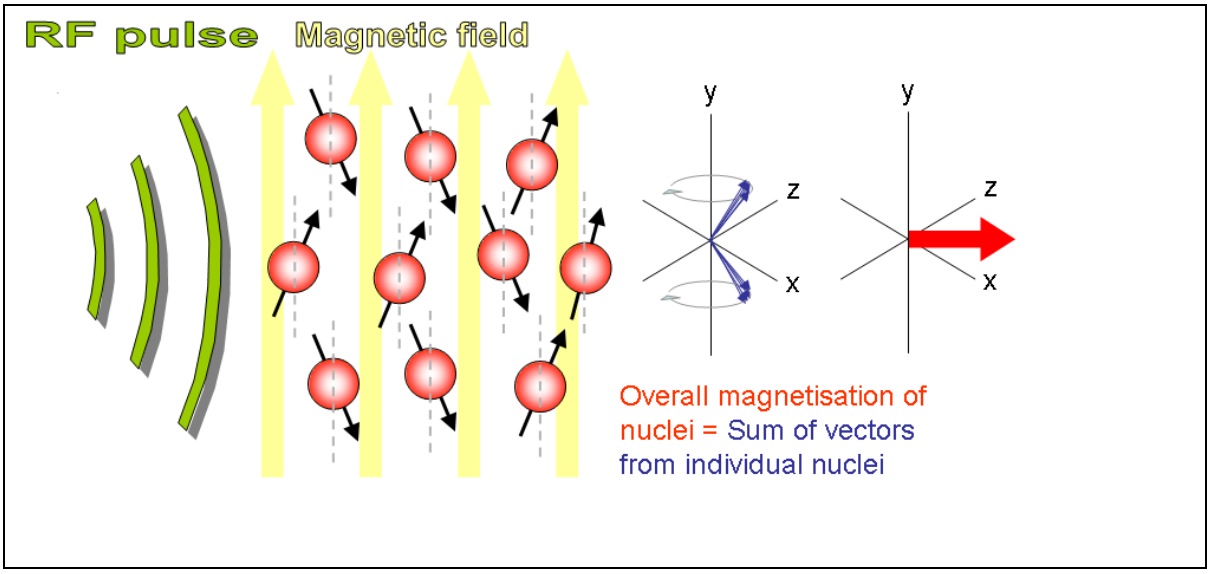


Figure 1-9: Graphical representation of the result of the application of a radiofrequency pulse to protons in a magnetic field, where protons precess together and a net magnetisation vector is produced.

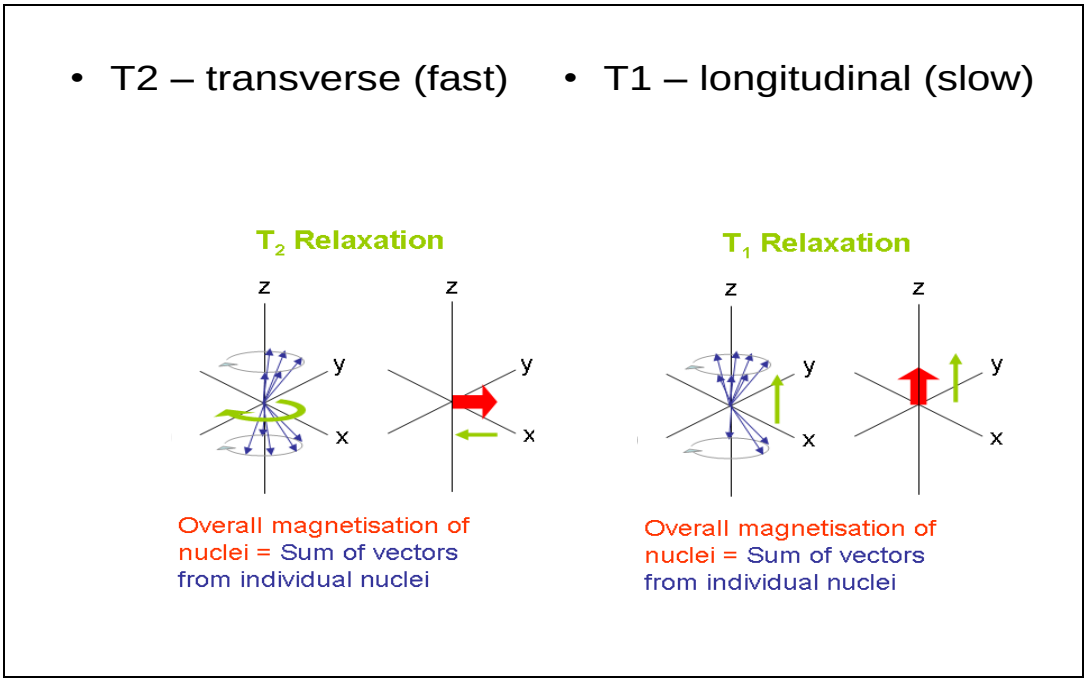


Figure 1-10: Graphical representation of T1 (longitudinal) and T2 (transverse) relaxation.

Cardiac MRI (CMR) utilises the ECG trace to time RF pulses, ensuring that each image is acquired at the same stage of the cardiac cycle. Gadolinium acts as an extracellular contrast agent and all current CMR contrast agents contain gadolinium. The recent recognition of gadolinium contrast induced nephrogenic systemic fibrosis has limited the use of this contrast agent to patients with an eGFR of >30ml/min/1.73m^2 [199]. Prior to this, gadolinium contrast agents were central to the redefinition of uraemic cardiomyopathy, whereby the marked increase in left ventricular mass in patients with renal disease was noted to be due not only to myocyte hypertrophy, but also due to diffuse fibrosis, as evidenced by late gadolinium enhancement(200).

1.8.2 Determinants of left ventricular mass in CKD

1.8.2.1 Gender

After adjustment for body surface area, left ventricular mass remains higher in men compared with women. Definitions of LVH take into account sex specific variations.

1.8.2.2 Systolic blood pressure

In patients receiving maintenance haemodialysis, we have shown systolic blood pressure to be the main determinant of LVH (201). Systolic blood pressure is a measure of the afterload. Afterload is determined by inherent wall properties (arterial stiffness), neurohumoral factors such as sympathetic stimulation, vasoconstriction and drugs. Increasing afterload increases strain on the heart resulting, via Starling's law, in an increase in myocardial stretch which over time leads to myocyte hypertrophy. Pulse wave velocity, a marker of arterial stiffness, which contributes to the afterload has been shown to correlate with SBP and left ventricular mass in patients with ESRD(202).

1.8.2.3 Level of renal function

Studies using echocardiography (198;203) have found a step-wise increase in the prevalence of left ventricular hypertrophy with declining renal function (CKD stages 1-4).

1.8.2.4 Proteinuria

The presence of microalbuminuria has also been shown to be predictive of LVH in patients with essential hypertension using echo(204;205) and ECG criteria(206) and also in patients with diabetes(207) without overt nephropathy. The relationship between proteinuria and

left ventricular mass index in patients with chronic kidney disease is however unclear. The MONICA/KORA study demonstrated using echo that albuminuria, even at low levels, was a significant predictor of LVMI (208). Paoletti et al recruited 244 patients with non-diabetic kidney disease stages 1-5 and calculated LV mass using echo and proteinuria with 24h collection. In the regression model LVMI was associated only with pulse pressure, proteinuria and duration of hypertension. When only patients with CKD 3-5 were considered, LVMI was associated with pulse pressure and age only – the association with proteinuria was lost. Using ECG Cornell voltage criteria, Nobakhthagighi(209) et al found an association between urinary albumin excretion, LVM and mortality, which they postulated may be due to diffuse microvascular injury.

1.8.2.5 Steroids

The impact of steroids on left ventricular mass has not been directly examined in patients with CKD. In EH, LVMI, as determined by echo, was shown to correlate significantly with morning plasma Aldo levels in a small group of untreated hypertensives off medications. Plasma Aldo levels were within the normal range and the majority of patients had LVH. The relationship persisted after adjustment for blood pressure(210). LVH has been reported in Cushing's disease as has dilated cardiomyopathy, often in the context of glucocorticoid driven hypertension(211). A small study of patients with hypertension without known glucocorticoid excess demonstrated a correlation between 24h urinary cortisol and LVMI. This was not seen in normotensive individuals. Of some concern, there was only a borderline correlation between 24h blood pressure and LVMI in that study(212).

1.8.2.6 Sodium

Cross-sectional analyses have reported an association between increased dietary sodium intake (as evidenced by increased urinary sodium excretion) and left ventricular mass(213). Others have demonstrated a reduction in LV mass with sodium restriction(214). In contrast, the Framingham Offspring study found no relationship between a spot urinary sodium:creatinine ratio and LV mass(215).

It is clear that patients with CKD have increased left ventricular mass compared with the general population and that by the time of commencing RRT over 50% will have left ventricular hypertropthy. However, the determinants of left ventricular mass in CKD have not been closely studied and therefore approaches to limiting LVH in these patients are guided by approaches used to treat hypertension. It is not unreasonable to suppose that

there may be factors specific to CKD which are not addressed by this approach and this will be studied further in this thesis.

1.9 The vasculature in chronic kidney disease

Large and small vessel function is altered in chronic kidney disease.

1.9.1 Pulse wave velocity and augmentation index – physiological concept

The arterial tree is as a visco-elastic tube which permits both forward and retrograde pressure waves. Cardiac contraction results in the forward wave, with the branch points and high resistance of the distal vessels generating retrograde waves. The compliance of the vessel is a product of the distensibility of the vessel wall and the blood volume contained within (216). The stiffer the artery, the faster the forward and retrograde wave speed. Arterial pressure can be increased by higher ventricular ejection, increased heart rate, higher vascular resistance and earlier wave reflection.

Due to differences in the proportions of collagen and elastin(217), proximal arteries are more elastic and distal arteries stiffer. Pulse wave velocity, as a reflection of stiffness, is therefore higher in more distal arteries (Table 1-3).

Therefore, as the pulse travels peripherally the amplitude increases and it has a narrower systolic peak. At a normal heart rate in a normal young individual, the brachial pulse pressure will be 20-50% greater than blood pressure in the aorta. Brachial pressure is therefore not a good surrogate of aortic pressure (218).

As arterial stiffness increases or arteriolar constriction occurs (218), the speed of the reflected arterial wave increases. It arrives early in systole, augmenting the forward wave, boosting systolic pressure further and resulting in a proportionately lower diastolic blood pressure. This reflection of arterial stiffness – the degree to which the reflected wave boosts the forward wave - is termed the augmentation index (Figure 1-11).

Site	PWV (m/s)(219;220)
Ascending aorta	4-5
Abdominal aorta	5-6
Iliac / femoral	8-9

Table 1-3: Pulse wave velocity according to site.

From O'Rourke and Staessen 2002(221).

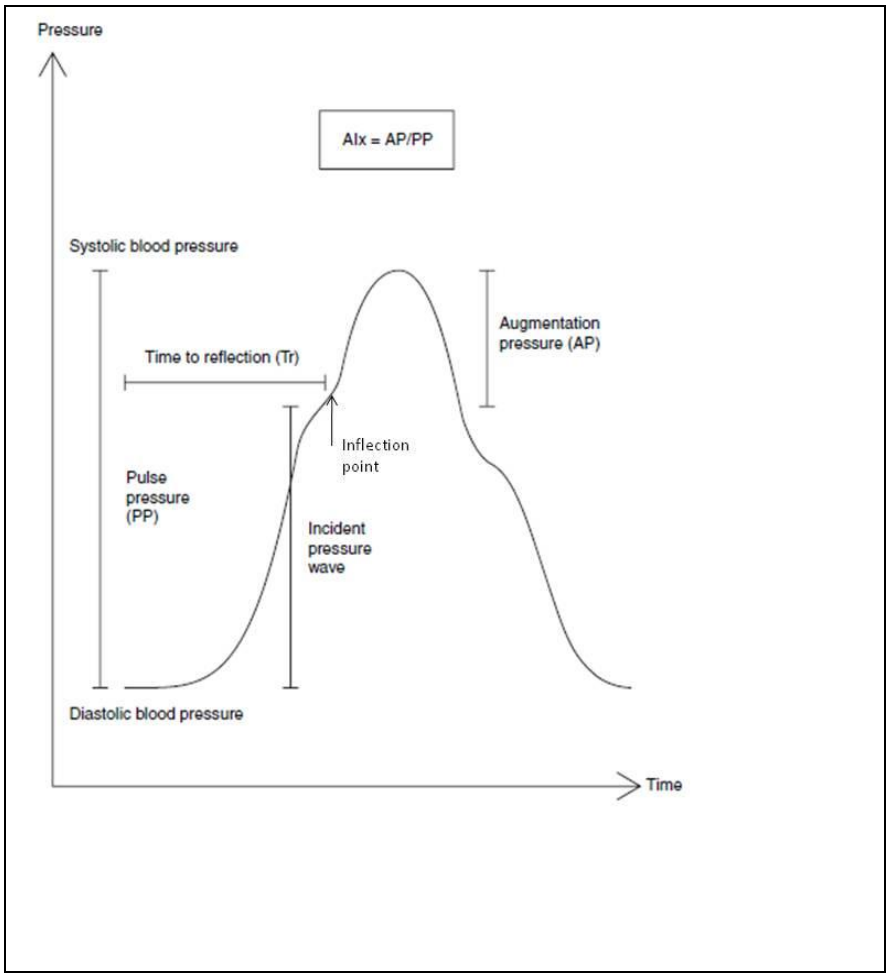


Figure 1-11: Graphical representation of the pulse wave and derived functions.

The augmentation index (AIx) is the augmentation pressure (AP) divided by the pulse pressure (PP). The augmentation pressure is the pressure difference between the maximal systolic peak and the inflection point (the merging point between the incident and reflected wave). Adapted from Janner et al(222).

Pulse wave velocity is derived from the distance between two recording sites in the line of pulse travel, and the time delay between corresponding points of the pressure wave which are not influenced by wave reflection – usually the initial upstroke(221). Carotid-femoral PWV is accepted as the gold-standard measurement of arterial stiffness(218) – its assessment is simple, non-invasive and reproducible.

1.9.2 Alterations in arterial structure in CKD

Traditional contributors to increasing arterial stiffness such as increasing age, diabetes, dyslipidaemia, hypertension and atherosclerosis are associated with CKD. However a number of other abnormalities also lead to the increased arterial stiffness seen in these patients. Vascular calcification, predominantly affecting the media, with transformation of smooth muscle cells to chondrocytes(223) contributes significantly. In addition, disordered bone metabolism, malnutrition, inflammation, oxidative stress fluid overload, renin-angiotensin system activation, insulin resistance and hyperhomocystinaemia are all thought to contribute to increased arterial stiffness(224).

The role of arteries in delivering blood to the periphery, as well as their role in cushioning the waveforms which are a consequence of ventricular ejection, are both affected by the remodelling which occurs in a uraemic environment due to the factors described above. Failure of the conductive role of arteries to provide sufficient blood to the tissues results in ischaemia. Failure to damp down the propulsive waveform, due to loss of elasticity, results in pulsatile surges in blood pressure, which in the kidney can lead to ‘barotrauma’ and glomerular damage. Increased arterial stiffness also contributes to a widened pulse pressure, left ventricular hypertrophy, increased myocardial oxygen demand, and impaired diastolic relaxation(225).

1.9.3 Determinants of AIx and PWV

In younger age, the PWV and velocity of the reflected wave are slow, therefore the wave returns during diastole. This relative proximal elasticity reverses with increasing age, in particular the stiffness of the carotid artery increases disproportionately when compared with the common femoral or radial arteries, which stiffen little. The stiffness of the common carotid artery of a 70 year old is 6 fold higher than that of a 20 year old(226;227).

The determinants of AIx are detailed in Table 1-4. AIx is higher in women than men, which is thought to reflect the smaller diameter of the vessels. Shorter height is associated

with a higher AIx due to less distance for the reflected wave to travel(228). Higher DBP, higher AIx; increasing age is associated with a higher AIx until the age of 60, but after the age of 60 DBP falls(222). Increasing heart rate reduces AIx due to a shorter systolic ejection period resulting in less overlap between forward and reflected waves(229). When a gender specific approach is adopted, AIx has been shown to correlate with established cardiovascular risk factors and the degree of coronary artery disease(222). An increasing augmentation index causes increased left ventricular strain and reduced diastolic coronary perfusion.

Factors associated with higher AIx	Factors associated with lower AIx
Female gender	Increasing heart rate
Shorter height	
Higher diastolic blood pressure	
Increasing age	

Table 1-4: Determinants of the augmentation index.

1.9.4 PWV and AIx – correlation with pathological outcome

Aortic stiffness increases progressively as renal function declines(230;231), even when eGFR is within the normal range(232), and has been associated with the urinary albumin:creatinine ratio in diabetic patients. PWV has been shown to be predictive for cardiovascular events and mortality in the general population(233;234) and those with end stage renal disease(235-237). On cross-sectional analysis of carotid-femoral PWV, there was a linear relationship between aortic stiffness and the severity of angiographically defined coronary artery disease in patients with varying stages of CKD(238), and similarly between PWV and electron beam computerized tomography coronary calcification score in CKD patients(239). PWV has also been shown to be an independent predictor of loss of GFR in patients with CKD(240-242).

The AIx has similarly been correlated with outcome. London et al demonstrated that each 10% increase in AIx was associated with a 50% increase in the relative risk of mortality in patients with ESRD(243). Takenaka et al demonstrated that a higher AIx is associated with decline in renal function in patients with relatively well preserved renal function(244). Similarly, Taal et al found an AIx above the group median to be associated with a 17.5 fold increased risk of requiring RRT in patients with CKD 4-5(240).

1.9.5 Endothelial function in CKD

The vascular endothelium has multiple roles including mediating coagulation, platelet adhesion and the immune response. Importantly, the endothelium also mediates vascular tone via production of vasodilators, particularly nitric oxide (NO) and prostacyclin. When the endothelium becomes dysfunctional, production of these vasodilators is reduced resulting in impaired vasodilatation. In atherogenesis, endothelial dysfunction and inflammation are important early interrelated steps(245). CKD is associated with an excess risk of cardiovascular disease not adequately explained by conventional risk factors and may be contributed to by endothelial dysfunction.

Whether endothelial dysfunction is a cause or consequence of renal disease is not clear. Endothelial dysfunction within the kidney may result in inflammation and oxidative stress which may be involved in the progression of CKD(246). However the endothelial dysfunction associated with CKD is not confined to the renal vascular bed, suggesting it may have a role in the systemic vascular disease associated with CKD.

1.9.6 Endothelial dysfunction measures and adverse outcome in CKD

Declining GFR has been shown to be associated with impaired endothelial function assessed using brachial flow mediated dilatation(247;248), circulating markers of endothelial function(249) and markers of oxidative stress(250). Dysfunction of the endothelium is central in the pathogenesis of a number of disorders such as haemolytic uraemic syndrome. Endothelial dysfunction is also known to exist in ESRD where patients exhibit an altered response to ischaemia, acetylcholine and hyperthermia (251).

1.10 Aims of this thesis

The aims of this thesis are as follows:

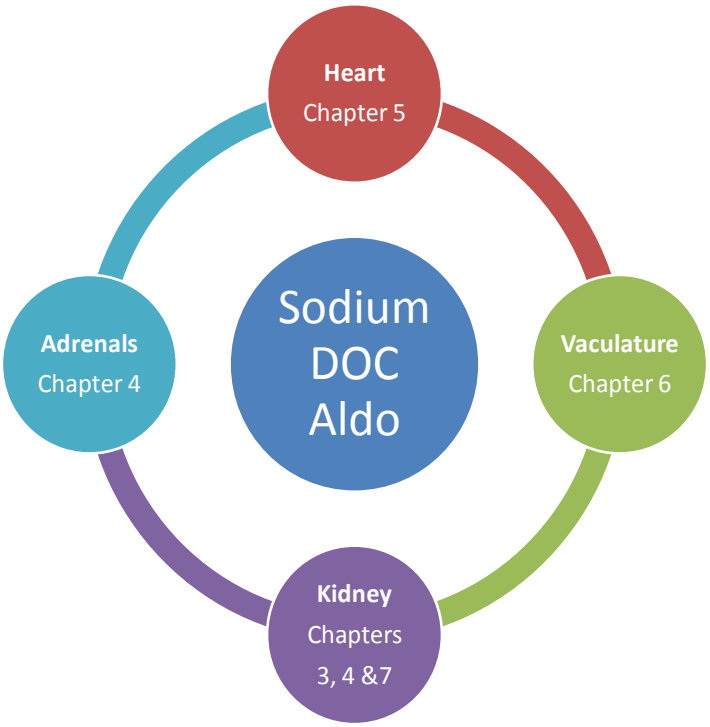
- To determine whether dietary sodium intake is associated with renal and overall patient outcomes.

- To ascertain whether mineralocorticoid or glucocorticoid levels are elevated in patients with chronic kidney disease, compared with patients with normal renal function.

- To assess the determinants of left ventricular mass and vascular function in patients with CKD.

- To determine whether elevated mineralocorticoid levels are associated with left ventricular hypertrophy, proteinuria excretion, endothelial dysfunction, pulse wave velocity and whether there is a synergistic relationship between endogenous mineralocorticoids, dietary sodium intake and pathological features.

- To ascertain whether human proximal tubules produce inflammatory or pro-fibrotic mediators when stimulated by steroids in a high sodium environment.



1-12: Summary of thesis – Interaction of sodium, DOC and aldosterone and effects on key organs and pertinent chapters.

1.11 Hypothesis

The hypothesis of this thesis is that chronic kidney disease is associated with proportionately higher levels of endogenous steroids than would be expected, which in association with a high dietary sodium intake, is associated with poor prognostic features (elevated LV mass, proteinuria, endothelial dysfunction and poor vascular function).

2 Chapter Two - Methods

2.1 Clinical Study

2.1.1 Ethics committee approval

The studies performed in this thesis were approved by the West Glasgow Research Ethics Committee. All subjects gave informed consent utilising forms approved by the committee (Appendix 1) after reading and digesting the patient information sheet (Appendix 2). The patient's general practitioner was informed of their participation in the study (Appendix 3).

2.1.2 Subjects and exclusion criteria

Adult patients with chronic kidney disease stage 3 or 4 (eGFR 15-60ml/min) were recruited from renal clinics in the West of Scotland. Patients were included if they had diabetic nephropathy, IgA nephropathy or membranous nephropathy. The diagnosis of diabetic nephropathy was clinician defined in the majority of patients – i.e. patients with known diabetes mellitus (type 1 or 2), microvascular complications such as proliferative retinopathy, proteinuria and an absence of alternative explanation for their renal failure. A minority had undergone native renal biopsy. Diagnosis of IgA nephropathy and membranous nephropathy were made from native renal biopsy. Patients with hypertension requiring drug treatment, ECG evidence of LVH and normal kidney function were recruited as control subjects from hypertension clinics at the Western Infirmary Glasgow.

Patients were excluded if they had a condition which would interfere with aldosterone metabolism or conditions which would make ability to complete follow-up unlikely. These were a known aldosterone secreting tumour or adrenal hyperplasia, treatment with an aldosterone antagonist (spironolactone or eplerenone) and patients who were pregnant or lactating. Known drug abusers and patients with an active psychiatric condition were excluded. Exclusion criteria for CMR scanning were implantable ferromagnetic devices (n=0), pacemaker (n=1), obesity (weight >130Kg or waist circumference greater than 120cm) (n=2) or claustrophobia (n=10). Membranous nephropathy patients receiving active immunosuppression other than low dose maintenance cyclosporin (<100mg/day) and prednisolone (≤ 5mg/day) were excluded. Cyclosporin does not interfere with aldosterone levels. There was no proteinuria cut off for renal patients.

2.1.3 Study design

Subjects visited the Glasgow Clinical Research Facility at 0900 on the day of assessment. For the preceding 24 hours patients performed a 24 hour urine collection. They were advised to collect all urine during the 24h period, after bladder emptying first thing in the morning, finishing the collection on the morning of assessment. From this, three aliquots of 20ml urine were stored at -80°C for urinary steroid analysis. The remainder was sent for routine laboratory measurement of urinary protein and electrolytes. A history was taken to ascertain current health, medications, smoking history and history of cardiovascular disease. Simple anthropometric measurements of height, weight and waist circumference were made. The patients then underwent more detailed phenotyping during the same visit.

2.1.4 Blood pressure measurement

After 15 minutes of supine rest, blood pressure was measured three times using the Omron MX2. An average of the three readings was obtained and used in further analysis. A minority of patients completed 24h blood pressure monitoring with the Tracker NIBP2/90217 device. During the day readings were taken every 15 minutes and every 30 minutes overnight.

2.1.5 Blood sampling

After 30 minutes of supine rest, 40ml of blood was taken for routine biochemistry (urea and electrolytes, bicarbonate, bone profile, liver function tests, C-reactive protein, urate, lipids and parathyroid hormone) and haematology (full blood count); plasma samples were obtained for aldosterone and renin measurement and stored at -80°C until analysis. An EDTA sample was obtained for genotyping.

2.1.6 Electrocardiogram

A standard 12-lead ECG was recorded at 25mm/s and 1mV/cm standardisation. The ECG was assessed as abnormal if the patient was not in sinus rhythm, had pathological Q waves, evidence of LVH (Sokolow-Lyon: S in V1 and R in V5 or V6 ≥35mm; R in aVL≥11mm), bundle branch block (QRS > 120ms), T inversion or ST depression >1mm.

2.1.7 Vascular stiffness

2.1.7.1 SphygmoCor Vx

In this study vascular function was measured non-invasively using the aortic augmentation index (AIx) and gold-standard carotid-femoral pulse wave velocity (PWV). The SphygmoCor® Vx system (Atcor Medical, Sydney, Australia) was used which utilises a Millar tonometer and the method of applanation tonometry (Figure 2-1). This has been validated in patients with CKD(238).

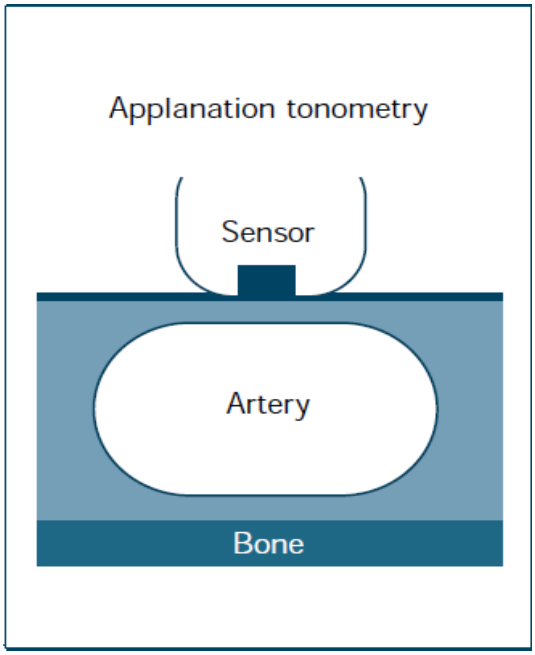


Figure 2-1: Demonstration of the principle of applanation tonometry.

The artery is compressed against a rigid structure, e.g. bone, and the tonometer senses alterations in pressure as each wave is propagated. The tonometer records these waves which are fed back to the computer for analysis.

Measurements were performed after an ECG, to ensure sinus rhythm, and blood pressure measurement. Measurements were standardised as follows(216):

- All usual drugs were continued prior to measurement.
- Patients were requested to refrain from smoking for at least 3h before measurement as smoking increases sympathetic tone and arterial stiffness.
- Room temperature was 21-23°C to standardise peripheral vasoconstriction
- Patients were assessed fasted as eating decreases systemic vascular resistance
- All patients were assessed at the same time of day as there is diurnal variation in arterial diameter with vessels being larger at night
- Patients were semi-recumbent for at least 10 minutes

2.1.7.2 Contraindications

Under certain conditions PWA / PWV cannot be performed (non-sinus rhythm, aortic valve disease). In this study two patients in atrial fibrillation were excluded.

2.1.7.3 Acquisition and analysis of augmentation index

The augmentation index was measured at the right radial artery, with the wrist dorsiflexed. Once an acceptable waveform was obtained, recordings were taken for a 15 second period, then waveforms manually inspected. The operator index, which is a composite of individual quality indices (pulse height variation <5%, diastolic variation <5%, shape variation <5%, average pulse height >100units), was reviewed and deemed acceptable if above 80%. Three readings were obtained for each subject and an average taken.

Augmentation index was recorded on three occasions and an average taken. The arterial pressure waveform is analysed from a derived central waveform – the radial waveform is converted mathematically to derive the central waveform using Fourier transformation. Analysis was performed using the calculated augmentation index (AIx@75bpm), which is corrected to a heart rate of 75bpm, allowing more standardised comparison (Figure 2-2).

Figure 2-2: Example of measurement of the augmentation index.

In the upper left hand panel green dots identify the systolic upstroke. The white trace is the radial waveform and the yellow trace the derived central (aortic) waveform. In the quality control section (top right panel) the values are white or green and therefore are all acceptable. The overall operator index is 100%. The aortic AIx (augmentation pressure / pulse pressure) is 20% and once adjusted for a heart rate of 75bpm, 15%.

2.1.7.4 Acquisition and analysis of pulse wave velocity

The principle of pulse wave velocity is graphically demonstrated in Figure 2-3. Three ECG leads were connected to the patient's chest. The ECG recording was manually inspected to ensure the R wave was upright and that the patient was in sinus rhythm. The right carotid artery pulse was located and the distance from this to the suprasternal notch was measured in millimetres. The right femoral artery was then located and the distance from the suprasternal notch to femoral artery, via the umbilicus, was measured in millimetres. To calculate the overall length, the values were entered into the SphygmoCor Vx, which uses a subtraction method to calculate length. This is distal distance (sternum to femoral) minus proximal distance (sternum to carotid). Pulse wave analysis was performed at the right carotid and right femoral arteries with the patient supine. Three readings with a standard deviation of less than 10% of the mean were obtained for each subject. Measurements were performed by the researcher or one of three supervised trained research nurses.

PWV = Distance (metres) / time (seconds). Figure 2-3 demonstrates the principles.

The SphygmoCor Vx measures the time between the R wave of the ECG (ventricular systole) and foot of the pressure wave at each site. Time between the R wave and proximal upstroke (carotid) is subtracted from the time between the R wave and distal upstroke (femoral), to obtain the transit time. Transit time is time of travel of the foot of the wave over a known distance. Pulse wave velocity was recorded in m/s and an average of 3 readings was used in analysis (Figure 2-4).

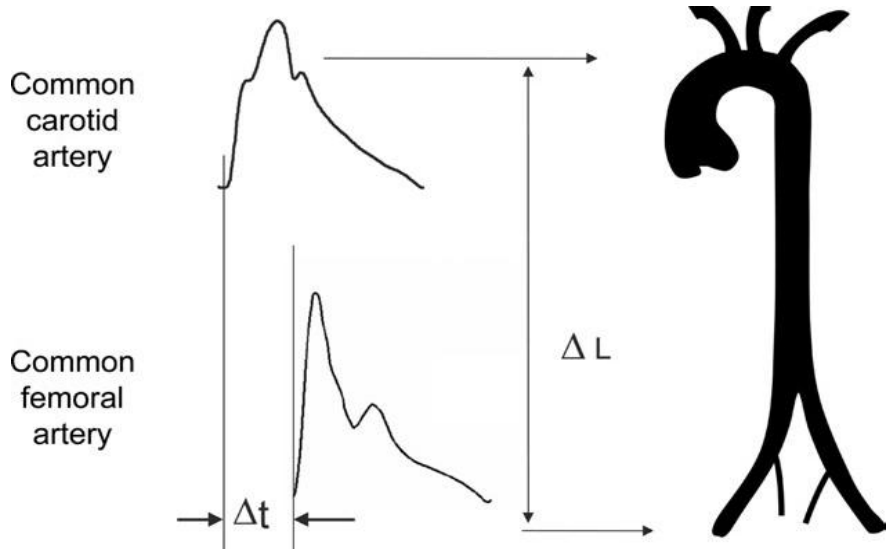


Figure 2-3: Ascertainment of the pulse wave velocity.

Δt is the difference in time between the upstroke in the common carotid artery and the upstroke in the common femoral artery, after the R wave. ΔL is the distance the pulse wave has to travel.

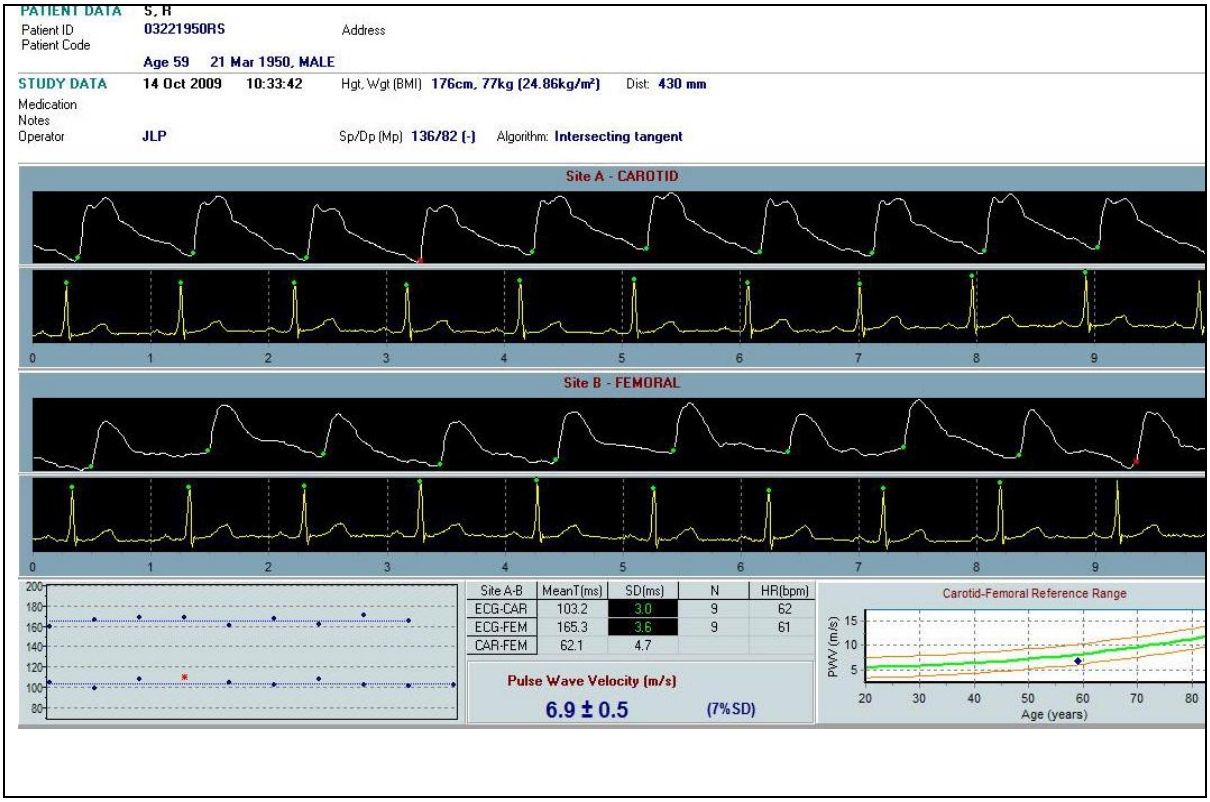


Figure 2-4: Example of measurement recording for carotid-femoral pulse wave velocity.

The green dots highlight the ECG R wave (ventricular systole) and the foot of the systolic wave at the carotid (upper panel) and femoral (lower panel) pulse. From these measurements, the times of 103.2ms and 165.3ms were determined. These readings were acceptable as the standard deviations (3.0, 3.6) were coloured green. The time difference of 62.1ms, together with the distance (430mm) results in a PWV of 6.9m/s. The SD was 0.5, which is 7% and therefore acceptable.

2.1.7.5 EndoPAT 2000 Principles

In this study, endothelial function was assessed using changes in Peripheral Artery Tone (PAT) volume by use of the Endo-PAT2000 system. This method is a quick, non-invasive, operator independent technique of assessing digital pulse amplitude during reactive hyperaemia, after a period of vascular occlusion. The subject acts as his own control, with endothelial function being tested in one arm, with the contralateral arm serving to monitor systemic vascular changes.

The principle of flow mediated dilatation is that ischaemia is induced (e.g. arterial occlusion), followed by restoration of blood flow, causing reactive hyperaemia. The increased flow results in increased shear stress which causes endothelium dependent vasodilation. The gold standard uses assessment of this dilation by ultrasound at the brachial artery but is highly operator dependent and requires extensive training. Endo-PAT uses the PAT technique to measure changes in the finger. The method measures nitric-oxide mediated vasodilation, in that the vasodilatory response was shown to be blocked by the administration of L-NAME (a nitric oxide inhibitor) (252). Studies have demonstrated close correlations between FMD and PAT methods(253;254).

The PAT signal is measured from the fingertip by recording finger arterial pulsatile volume changes. The measurement apparatus utilises modified plethysmography sensors. The probes also impart a uniform subdiastolic pressure to the distal two thirds of the finger tips to prevent distal venous blood pooling, increasing the signal to noise ratio (Figure 2-5).

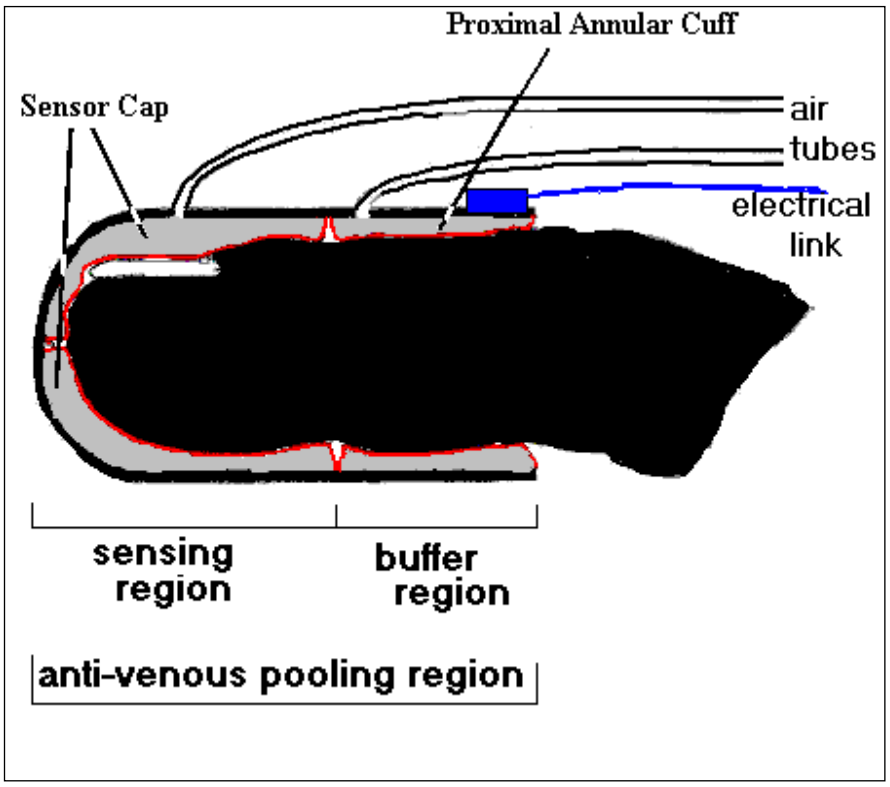


Figure 2-5: PAT technology – probe on index finger.

The EndoPAT system has been extensively validated. EndoPAT has been correlated with coronary artery endothelial function assessed by intra-coronary acetylcholine infusion(255). An EndoPAT reactive hyperaemia index cut-off of 1.67 provided a sensitivity of 82% and specificity of 77% for diagnosing coronary endothelial function. EndoPAT was validated in the Framingham cohort(256), where nearly 2000 subjects were assessed, and the reactive hyperaemia index (RHI) was been shown to be inversely correlated with male sex, body mass index, cholesterol, diabetes, smoking and coronary artery disease(253;255;256), but not blood pressure. The utility of EndoPAT has not been examined in patients with chronic kidney disease.

2.1.7.6 EndoPAT 2000 contraindications

Patients with Raynaud's or arterio-venous fistulae would be excluded, however no such persons were identified in this study.

2.1.7.7 EndoPAT 2000 acquisition and analysis

The fasted subject lies semi-recumbent in a quiet room with a temperature of 21-23°C. The probes are fitted onto both index fingers and baseline readings are taken for 5 minutes. A blood pressure cuff is then inflated over the brachial artery for 5 minutes to 200mmHg, or 60mmHg above systolic pressure. Following the 5-minute supra-systolic occlusion period, readings in the reactive hyperaemia phase are recorded for another 5 minutes.

Analysis is automated using software provided by Itamar medical. The RHI is calculated as a ratio of the average amplitude of PAT i.e. the ratio of the post-deflation pulse amplitude to baseline pulse amplitude in the intervention arm is normalised to the control arm (Figure 2-6). Endothelial dysfunction results in a lower peripheral hyperaemic response.

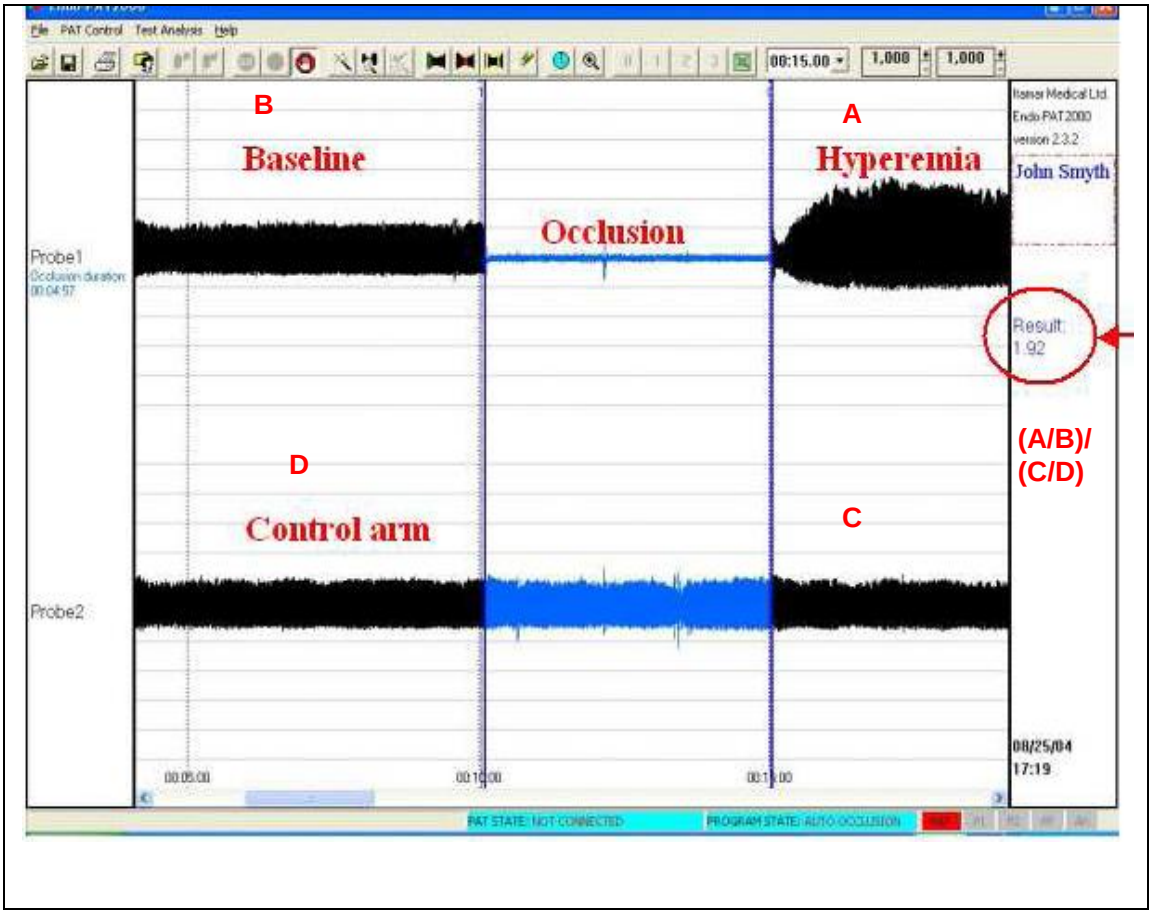


Figure 2-6: Pictoral demonstration of PAT signal in both arms during the three phases of the test with demonstration of how the RHI is calculated.

Probe 1 records the intervention arm and probe 2 the control arm. Sections B&D show the signal over the initial 5 minutes. The second 5 minutes (blue signal) demonstrates no flow through the finger of the intervention arm, whilst the cuff is inflated. The third 5 minutes shows an increase in flow through the intervention arm finger (A) compared with baseline (B) and control (C). The RHI result is calculated from (A/B) / (C/D).

2.1.8 Cardiac Magnetic Resonance Imaging

2.1.8.1 Principles

Magnetic resonance imaging (MRI) maps proton density (hydrogen nuclei) by utilising the proton spin in a high strength magnetic field with the application of radiofrequency pulses. The application of a radiofrequency pulse results in a proton net magnetization vector for the duration of the pulse. Once the RF pulse ceases, the protons will return to their natural state, emitting energy. This emitted energy generates a voltage and is measured using a coil.

2.1.8.2 Features specific to cardiac imaging

RF pulses of varying duration or strength and the application of magnetic-field gradients, that are adjusted to highlight desired tissue characteristics, form a pulse sequence. Basic pulse sequences used in CMR are spin-echo and gradient-echo sequences. Spin echo sequences are often used for assessment of cardiac morphology and flowing blood normally appears black. In gradient echo sequences, flowing blood is represented by high signal intensity and turbulences as areas of signal void – utilised for assessing valvular lesions, shunts and great vessels. In this study, the TrueFISP (fast imaging with steady-state precession) sequence was used. This is the Siemens pulsed gradient-echo sequence.

Cardiac gating uses the ECG tracing to trigger the radiofrequency signal, meaning that each image is always acquired at the same phase of the cardiac cycle so that phase mis-mapping from cardiac motion is reduced. Respiratory motion artefact is minimised by breath holding.

2.1.8.3 Utility

Cardiovascular magnetic resonance imaging (CMR) is a non-invasive method used to accurately define ventricular dimensions and is accepted as the ‘gold standard’ for assessing left ventricular mass and function. Echocardiography derives LV mass and dimensions from one- or two-dimensional data, using assumptions of cardiac geometry, unlike CMR where actual myocardial mass and volumes are obtained. CMR does not utilise ionising radiation and therefore has advantages over techniques such as computed tomography. In comparison to echo scanning, assessment of the LV dimensions is ‘volume

independent', which is extremely attractive in patients with chronic kidney disease who are prone alterations in intravascular volume status.

2.1.8.4 Contraindications

Patients are unable to enter the scanner if they have implanted ferromagnetic objects. Intraocular shards of metal e.g. former welders and intracerebral clips are contraindications. A cardiac pacemaker or implantable defibrillator remains an absolute contraindication to CMR scanning. One patient was excluded on this basis. Patients with claustrophobia may not tolerate study with CMR – ten patients were excluded on this basis. Lastly, extreme central adiposity or weight >130Kg is a contraindication as patients are physically unable to enter the scanner (n=2). Prior to entering the scanner patients are assessed using a standard MRI safety screening questionnaire.

2.1.8.5 Image acquisition

Non-contrast CMR was performed using a 1.5 Tesla Siemens (Erlangan) cardiac MRI scanner. All scanning was performed by the investigator, with supervision by a cardiac radiographer (Ms. Tracey Steedman, Glasgow CMR Unit, Western Infirmary).

2.1.8.6 Patient positioning

The patient lies supine and enters the scanner head first. Patients are connected to ECG leads and the cardiac chest coil. Headphones are used to relay breath holding instructions.

2.1.8.7 Left ventricular mass and function

Each set of images are acquired using repeat breath-holds (duration approximately 8 seconds each) and retrogated to the electrocardiogram. Each scan takes approximately 30 minutes. No contrast agents/drugs are required for the scan.

Initial localizers are performed using long-axis pilot scans followed by a fast imaging with steady-state precession (FISP) sequence, which was used to acquire cine images in long-axis planes (vertical long axis, horizontal long axis, LV outflow tract) followed by sequential short-axis LV cine loops (8-mm slice thickness, 2-mm gap between slices), from the atrioventicular ring to the apex. Slices were contiguous.

The complete protocol is as follows:

1. Initial multi-slice localiser in end expiration.

2. Using an axial image of the left ventricle, the vertical long axis localiser (VLA) is obtained through the apex of the left ventricle, aligning it with the centre of the mitral valve (Figure 2-7).

3. From this image, the four chamber horizontal long axis localiser (HLA) is planned through the mid-point of the mitral valve and apex of the left ventricle.

4. The HLA localiser is used to produce three short axis (SA) localiser slices with orientation parallel to the mitral valve plane through the atrioventricular groove.

5. The SA images are then utilised to plan three long axis cine views – the 4-chamber, 2-chamber and left ventricular outflow tract (LVOT) views.

6. From the cine 4-chamber study serial 8-mm-thick short-axis images were obtained. The first image was positioned in orientation across the mitral valve plane using the atrioventricular groove to orientate (Figure 2-8).

Imaging parameters, which were standardized for all patients, included repetition time/echo time/flip angle/voxel size/field of view of 3.14 ms/1.6 ms/60°/2.2 _ 1.3 _ 8.0 mm/340 mm.

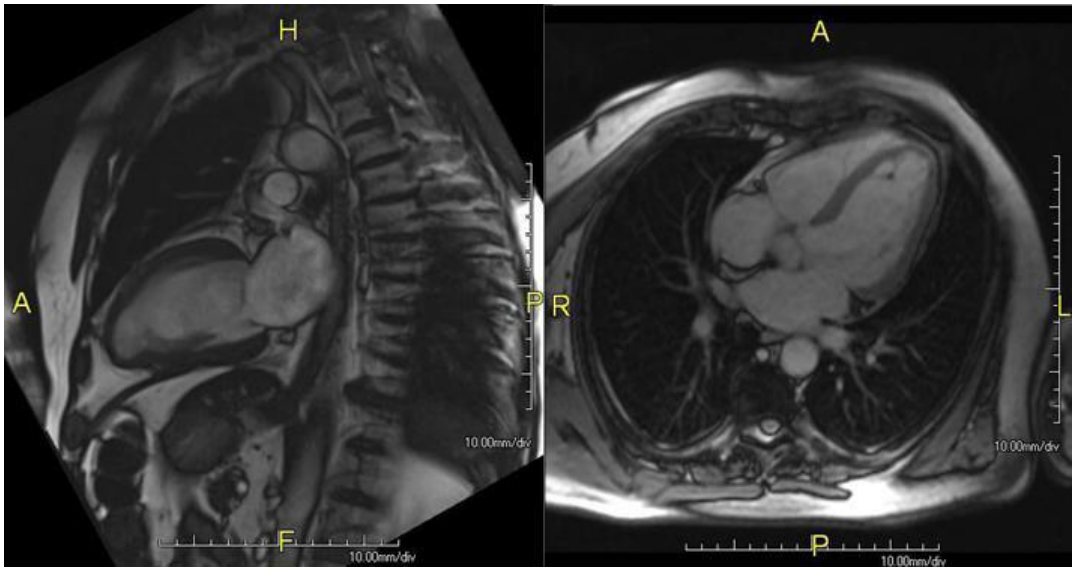


Figure 2-7: Left panel – vertical long axis view. Right panel – horizontal long axis view.

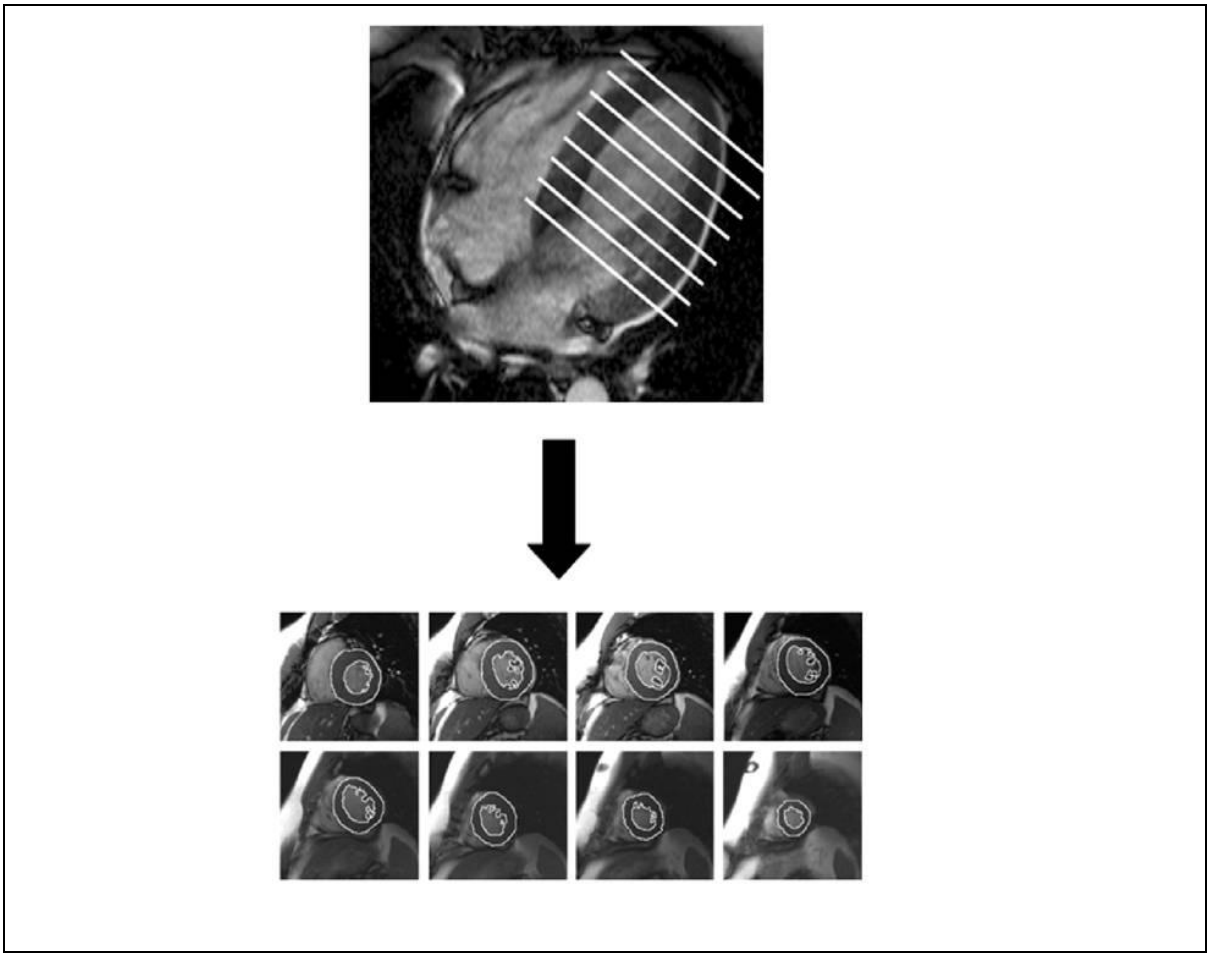


Figure 2-8: Axial slices through the horizontal long axis cine in maximal diastole producing a short axis cine stack, with measurements performed for Argus analysis.

2.1.8.8 Image analysis – Left ventricular mass and function

Left ventricular mass and function was analysed from the left ventricular short axis cine loops using manual tracing of epicardial and endocardial end-systolic and end-diastolic contours. End-systolic volume (ESV), end-diastolic volume (EDV) and LVM were calculated using analysis software (Argus; Siemens). Values were adjusted for body surface area using the Mosteller formula:

Body surface area (m^2) = √((weight (Kg) x height (cm))/3600))

Left ventricular ejection fraction was calculated as:

LVEF = ((EDV-ESV)/EDV) x 100

Left ventricular abnormalites were defined as follows (Table 2-1) using published mean normal LV dimensions for healthy volunteers plus two standard deviations(257). Analysis of left ventricular mass and function required 10 minutes per patient.

Left ventricular abnormality	***Measure***	***Male***	***Female***
LVSD	LVEF	<55%	<55%
LVH	LVMI (LVM/BSA)	>84.1g/m^2	>66.3g/m^2
LV dilatation	EDV/BSA	>111.7ml/m^2	>99.3ml/m^2
LV dilatation	ESV/BSA	92.8ml/ m^2	70.3ml/ m^2

Table 2-1: Definitions of left ventricular abnormality, subdivided by gender.

2.1.9 Plasma analysis

2.1.9.1 Routine biochemistry and haematology

Samples were sent to biochemistry for analysis of urea and electrolytes, bicarbonate, bone profile, liver function tests, C-reactive protein, urate, lipids and parathyroid hormone (Diasorin Liason). Full blood count was measured routinely by haematology.

2.1.9.2 Aldosterone

After 15-20 minutes of supine rest, 5ml of blood was withdrawn into a ZZ tube and then spun at 2500RPM for 10 minutes, with the plasma frozen at -80°C. Aldosterone was measured in batches by the biochemistry department utilising a radioimmunoassay (Siemens, TKAL2, Coat-A-Count). Normal range for a supine adult: 100-400pmol/l.

2.1.9.3 Renin

After 15-20 minutes of supine rest, 4ml of blood was withdrawn into a potassium EDTA tube (1mg/ml) and then spun at 2500RPM for 10 minutes, with the plasma frozen at -80°C. Plasma renin concentration was measured in the routine biochemistry laboratory using the DIASORIN analyser. Normal range for a supine adult: undetectable-40mIU/l.

2.1.10 Urine analysis

2.1.10.1 Sodium and creatinine

24 hour urine collection was collected in a bottle without preservative. Urine was analysed in the routine laboratory for electrolytes using flame photometry.

2.1.10.2 Urinary steroid analysis

After completion of the 24h urine collection, urine volume was noted and three 20ml aliquots of urine were collected in plain universal containers and stored at -80°C. 24-hour excretion rates of each steroid were determined by gas chromatography-mass spectrometry using the Shackleton method(258) (performed by Ms. Mary Ingram, MRC Blood Pressure Group, British Heart Foundation Cardiovascular Research Centre). Urinary free cortisol and cortisone were measured by Dr. Stephen Miller (MRC Blood Pressure Group, British Heart Foundation Cardiovascular Research Centre) utilising a high performance liquid chromatography assay(259). Table 2-2 describes measured metabolites.

Abbreviation	Metabolite name	Full name	Urinary excretion reference range (g/24h)	Urinary excretion reference range (mol/24h)
THALDO	Tetrahydroaldosterone	3α,11β,21-trihydroxy-18-al-5β-pregnane-20-one	10-120 µg/24h	27-330 nmol/24h
THDOC	Tetrahydrodeoxycorticosterone	3α,21-dihydroxy-5β-pregnane-20-one	2-28 µg/24h	6-84 nmol/24h
THF	Tetrahydrocortisol	3α,11β, 17α,21-tetrahydroxy-5β-pregnane-20-one	310-3680 µg/24h	1-10 µmol/24h
ALLO THF	Allo-tetrahydrocortisol	3α,11β, 17α,21-tetrahydroxy-5 α-pregnane-20-one	490-3480 µg/24h	1-10 µmol/24h
THE	Tetrahydrocortisone	3α,17α,21-trihydroxy-5 β-pregnane-11,20-dione	1100-5100 µg/24h	3-14 µmol/24h
THS	Tetrahydro-11-deoxycortisol	3β,17 α,21-trihydroxy-5 β-pregnane-20-one	3-130 µg/24h	9-370 nmol/24h
F	Cortisol		3.5-45 µg/24h	9.7-124.2 nmol/24h
E	Cortisone		17-129 µg/24h	47.3-358.6 nmol/24h

Table 2-2: Table of urinary metabolites analysed, including abbreviation, full name and urinary excretion reference range in ug/24h and nmol/24h.

2.1.11 Genetic analysis

2.1.11.1 DNA extraction

DNA was extracted from whole venous blood (EDTA) samples, after refrigeration, using the Autopure LS (Large Sample Nucleic Acid Purification Instrument) by Gentra Systems. After DNA extraction the samples were quantified using a Nanodrop 1000 to give a 260/280 ratio (>1.6 acceptable) and a DNA value in ng/ml. This was performed by Mr. Jim McCulloch (Chief Technician).

2.1.11.2 SNP analysis

Aldosterone synthase gene -344C/T polymorphism genotyping was performed by Dr. Samantha Alvarez-Madrazo (Research Assistant). The region of DNA containing the –344 C/T polymorphism was amplified by polymerase chain reaction (PCR) using primers (Oswel DNA Service, Southampton, UK) (F 5′GTG TCA GGG CAG GGG GTA3′. R 5′AGG CGT GGG GTC TGG ACT3′). The 228 base pair product was digested by Hae III (Promega, Southampton, UK) and subject to electrophoresis in 3% MetaPhor agarose gel.

2.2 Laboratory project

2.2.1 Materials

2.2.1.1 Biochemicals

Unless otherwise stated all chemicals were obtained from Sigma, Poole, UK. Chemicals were stored at -20°C until usage and were diluted with media to the required concentration prior to use. Repeated freeze-thaw was avoided. Table 2-3 lists chemicals used.

Chemical	Company	Catalogue number	Molecular weight	Initial dissolving protocol
Aldosterone	Sigma-Aldrich	A9477	360.44	5mg diluted in 5ml ethanol and 9ml media to concentration 10^{-3} M
Cortisol	Sigma-Aldrich	C2505	346.46	0.034g was dissolved in 10ml ethanol to concentration of 0.01M.
Angiotensin II	Sigma-Aldrich	A9525	1046.2	0.001g was dissolved in 9.558ml of sterile water to concentration of 10^{-4} M
Canrenoic acid	Sigma-Aldrich	C7287	397.57	0.3975g were dissolved in 10ml of DMSO to a concentration of 100mM
Lipopolysaccharides	Sigma-Aldrich	L4005		1mg was diluted in 10ml PBS

Table 2-3: Chemicals used during laboratory project including source, molecular weight and initial dissolving protocol.

2.2.1.2 Immunochemicals

The antibodies used in this study are detailed in Table 2.4.

Protein target	Manufacturer	Species	Concentration
Collagen 1A	AbCam	Mouse	1/2000
Collagen 1A	Santa-Cruz	Mouse	1/200
pERK p44/42 MAP kinase	NEB	Rabbit	1/1000
Total ERK	NEB	Rabbit	1/1000
GAPDH	Abcam	Rabbit	1/1000
Anti-mouse HRP	Amersham	Sheep	1/2000
Anti-rabbit HRP	Amersham	Donkey	1/2000

Table 2-4: Immunochemicals used, manufacturer, species and concentration antibody utilised at.

2.2.1.3 Cell biology

Human kidney 2 (HK-2) cells are a proximal tubular epithelial cell line derived from normal adult human kidney (CRL-2190; American Type Culture Collection, Rockville, MD)(260) immortalized with the human papilloma virus 16 (HPV-16) E6/E7 (Figure 2-9). Cells were utilised between passages 3 and 25. Cells were incubated at 37°C in 5% CO_2 with keratinocyte serum-free medium (K-SFM; Life Technologies, Grand Island, NY) containing 1 mM glutamine, 5 ng/ml epidermal growth factor, 40 µg/ml bovine pituitary extract, 25 U/ml penicillin, and 25 µg/ml streptomycin. At near confluence, the cells were trypsinized and transferred to either additional flasks for passage or to plates at a minimum density of 2×10^5 cells for experimentation. Experiments were conducted 24 hours after passage, with the cells in a subconfluent state.

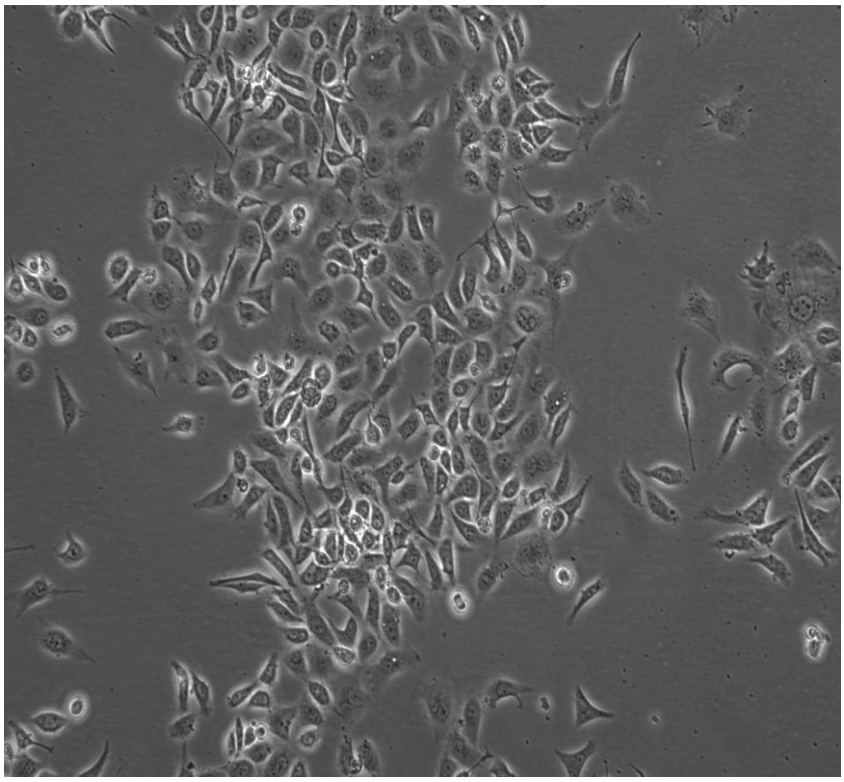


Figure 2-9: HK-2 cells under light microscopy.

2.2.2 Molecular biology

2.2.2.1 RNA extraction and complementary DNA synthesis

Cytoplasmic RNA was extracted from trypsinised cells using the Qiagen RNeasy Mini Kit (Qiagen). RNA concentration was determined by measuring absorbance at 260nm and purity estimated by checking the 260nm:280nm absorbance ratio using the Thermo Scientific Nanodrop 1000 spectrophotometer (Ratio >1.6). RNA was stored at -80°C. Complementary DNA was synthesised using the Invitrogen M-MLV reverse transcriptase kit. 1ug of RNA, 1ul hexamers (0.5ug/ul), 1ul 10 mM dNTP mix and sterile water to a volume of 12ul were heated to 65°C for 5 minutes and chilled on ice. Thereafter 4ul of 5x First-Strand buffer, 2ul 0.1M DTT and 1ul RNaseOUT (recombinant ribonuclease inhibitor) were added. Samples were incubated at 37°C for 2 minutes before adding 1ul of reverse transcriptase and incubating at 37°C for 50 minutes followed by 70°C for 15 minutes. Each sample was also amplified without reverse transcriptase to ensure there was no DNA contamination. Samples were subsequently diluted 1/5 to a final volume of 100ul with distilled water and stored at -20°C.

2.2.2.2 Polymerase chain reaction - Primer design

Intron spanning primers were designed using the Roche universal probe library (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000). Primers were ordered from Integrated DNA Technologies (Belgium). Table 2-5 lists primers and characteristics.

Target gene	Accession no.	Forward primer	Reverse primer	Ann. Temp	Probe no.	Amp. size
TGFβ1	NM_000660.3	gcagcacgtggagctgta	cagccggttgctgaggta	59	72	64
TGFβ2	NM_003238.1	ccaaagggtacaatgccaac	cagatgcttctggatttatggtatt	60	67	114
TGFβ3	NM_003239.2	aagaagcgggctttggac	gcgcacacagcagttctc	60	38	63
Collagen I	NM_000088.3	gggattccctggaccataaag	ggaacacctcgctctcca	59	67	63
Collagen IV	NM_001845.4	cgggtacccaggactcatag	ggacctgcttcacccttttc	59	81	60
Beta-actin	NM_001101.3	ccaaccgcgagaagatga	ccagaggcgtacagggatag	59	64	97
11BHSD2	NM_000196.3	gtcaaggtcagcatcatcca	cactgacccacgtttctcac	59	71	65
MCR	M16801.1	aacaggtagacggcgagaga	Tcagggagactgtggtagcc	60	58	73

Table 2-5: Oligonucleotide gene targets, NCBI NM accession number, oligonucleotide sequences (forward and reverse primers), optimal annealing temperature (Ann temp), paired Universal Probe number (Probe no.) and amplicon size (Amp. Size).

2.2.2.3 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using a PTC-100 programmable thermal controller. A 25ul reaction volume consisted: 1ul of template (0.2ng), 0.25ul 100nM forward and reverse primers, 0.5ml 10mM dNTPs, 0.25ul Taq polymerase, 5ul 5x buffer and PCR-grade water. The following thermal cycling profile was used for 35 cycles: Samples were denatured at 94°C for 30 seconds then primers annealed at 55°C for 45 seconds and DNA extension at 68°C for 1 minute.

2.2.2.4 Real time polymerase chain reaction

Real time PCR was used to determine relative quantification of mRNA transcripts following reverse transcription. The Roche Universal Probe Library (Roche) was used in a probe-based detection scheme. This method utilises a time sensitive quantification of PCR template (i.e. real time), as opposed to endpoint detection, with the fluorescent reporter signal being detected and quantified at the end of each cycle, with the signal increasing proportionally to the amount of PCR product in the reaction. A fixed fluorescence threshold is set above baseline (the threshold cycle (Ct)) which is the cycle number at which fluorescence emission exceeds threshold and is exponential.

The Taqman probe is an oligonucleotide which is shorter than primers. There is a fluorescent dye at the 5' base and a quenching dye on the 3' base. When the probe is intact, there is no emission of fluorescence. The probes are designed to anneal to a region of the PCR product. When polymerase replicates a template on which a Taqman probe is bound, the 5'exonuclease activity cleaves the 5' end of probe which contains the reporter dye (FAM) and the reporter emits fluorescence – increasing with each cycle proportional to the rate of cleavage. Accumulation of product is detected by monitoring increase in fluorescence compared with passive reference dye e.g. Rox.

An internal control gene, which is more abundant and remains constant in proportion to total RNA, is also amplified e.g. B actin. This allows normalisation of the mRNA target to take account of differences in total RNA added to each reaction. Relative quantification of gene expression is then undertaken using the comparative CT method, where the difference between the CT values of two PCRs for the same initial template amount varies. This is then normalised to a baseline reference sample for comparison to be made ($\Delta\Delta$CT method).

A 10ul well volume was composed of: 0.2ng of template (2ul), 0.1ul 100nM probe, 0.4ul of 100nM forward and reverse primers, 2.1ul PCR-grade water, 5ul QPCR mix with 120 nM ROX (reference dye). 384 well plates were plated and beta-actin was used as the housekeeping gene. Controls of non-reverse transcribed template and mastermix without template were also run to ensure there was no contamination. Using the TaqMan cycler, the block was heated to 50°C for 2 minutes then samples heated to 95°C for 10 minutes ('hot start') followed by 40 cycles of denaturing at 95°C for 15 seconds and 1 minute annealing / extension at 60°C. In order to assess specificity of amplified product and to avoid spurious results from primer-dimer formation and amplification, dissociation curves showing product melting points were assessed (Figure 2-10) and samples of product were run and visualised on agarose gels.

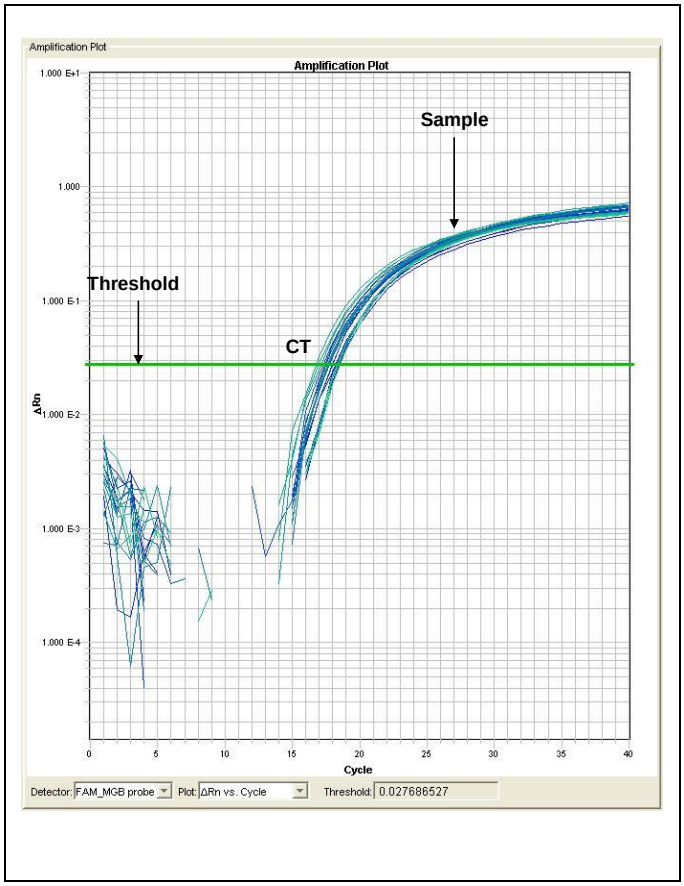


Figure 2-10: Amplification plot of normalised fluorescence intensity versus cycle number.

The threshold cycle (CT) is defined as the point when a statistically significant rise in the amount of DNA product is detected.

2.2.2.5 Agarose gel electrophoresis of DNA

DNA samples were mixed with 6x blue loading buffer (Promega) and run on agarose gels with tris-acetate (TAE) electrophoresis buffer (0.04M Tris-acetate, 0.001M EDTA). 2% agarose gels were made (0.8g agarose for molecular biology (Sigma), 40ml TAE buffer, 4ul ethidium bromide) and 15ul of sample loaded and run at 100V for 1hour. Bands were visualised on an ultraviolet transilluminator and images recorded on the BioRad ChemiDoc XRS digital imaging system.

2.2.3 Biochemical assays and methods

2.2.3.1 Preparation of cell lysates

Cells were lysed after trypsinisation using RIPA buffer supplemented with protease inhibitors (benzamidine and soya bean), orthovanadate and PMSF. Bio-Rad Protein assay was undertaken to quantify cell lysate protein concentration by measuring absorbance of Coomassie Brilliant Blue G-250 at 595nm. Bovine serum albumin standards were used to construct a standard curve. Protein concentrations of all samples were routinely measured in duplicate. Lysates were stored at -80°C until use.

2.2.3.2 Polyacrylamide gel electrophoresis and immunoblotting

Proteins were resolved using the NuPAGE 4-12% Bis-Tris precast gel system (Invitrogen). Minimum sample protein content was 10ug per well. Protein samples were denatured in NuPAGE LDS (lithium dodecyl sulphate) sample buffer by heating to 70°C for 10 minutes. In order to reduce proteins, 50mM dithiothreitol was added immediately prior to sample denaturing.

Proteins resolved by polyacrylamide gel electrophoresis were transferred to Protran nitrocellulose membranes using the NuPAGE XCell II blotting apparatus and manufacturer's transfer buffer which was supplemented with 20% methanol. Transfer was performed at 40V for 60mins. Transfer efficiency was assessed by staining membranes with Ponceau S solution followed by washing with TBS (20mM Tris-HCl pH 7.6, 135mM sodium chloride) containing 0.1% Tween 20 (TBST).

The nitrocellulose membranes were then blocked in 4% non-fat powdered milk (Marvel) for 1hour at room temperature. Blots were incubated with primary antibodies at the optimal concentration in milk overnight at 4°C. Three washes of 5 minutes with TBST were then

carried out prior to incubation with the appropriate horseradish peroxidise conjugated secondary antibody for 2 hours in milk at room temperature. Blots were then washed with TBST for 5 minutes 5 times. Proteins were visualised by enhanced chemiluminescence (Amersham) and autoradiography.

2.2.3.3 Enzyme linked immunosorbent assay (ELISA)

ELISA kits were purchased from BD Biosciences (San Jose, CA, USA) and ELISA conducted on cell culture supernatant. Human TGF-β1 (559119), human IL-6 (555220), human GM-CSF (555126) and human IL-8 (555244) were analysed. Recommended study procedure was followed using 96 well plates, with results read on the Wallac Victor2 1420 multilabel counter. Samples for GM-CSF analysis were diluted 1/5 at 24h and 1/10 at 48h. IL-6 and IL-8 were diluted 1/40 or1/80 at 24h and 48h respectively. One hour samples were run undiluted. Sample concentrations were calculated from standard curves and sample results normalised to the control well.

3 Chapter Three - A study of urinary sodium excretion and outcomes in patients with chronic kidney disease

3.1 Introduction

Sodium is the main extracellular cation and plasma sodium concentration is maintained within a narrow range by the kidney. Despite its vital physiological role and the numerous mechanisms maintaining sodium homeostasis, elevated dietary sodium intake has been shown to have negative effects. In large population based studies(54) sodium intake has been shown to influence blood pressure, with sodium restriction being associated with a fall in both systolic and diastolic blood pressure(56) (64). Similarly, sodium intake is widely believed to influence progression of CKD, independent of effects on blood pressure(63). There is experimental evidence to suggest a direct pathogenic role for sodium in renal failure(64), however hard endpoint data in humans are lacking (see introduction section 1.3).

Urinary sodium (UNa) excretion (mmol/24h) is the most reliable easily available method of assessing oral sodium intake, independent of dietary assessment. UNa mirrors dietary sodium intake in patients with CKD and reduced glomerular filtration rate (44), but difficulties might be anticipated with the interpretation of urinary sodium values in advancing kidney disease. As GFR declines appetite can be reduced, which might impact upon dietary sodium intake. Similarly, UNa is closely related to weight, and the impact of declining GFR on weight and muscle mass, and how best to adjust for these factors is not clear.

As such, the aims of this study were to assess:

- Levels of UNa in patients with impaired renal function.

- The impact of declining renal function on the fractional excretion of sodium (FENa), a reflection of the percentage of sodium filtered versus the percentage sodium reabsorbed.

- How best to report UNa excretion in patients with CKD - total urinary sodium excretion (mmol/24h) (UNa/24h) or UNa adjusted for urinary creatinine (UNa:Cr ratio).

- Whether urinary sodium excretion correlates with renal outcome or patient survival in patients with CKD, and whether higher levels of dietary potassium intake are protective in this population.

3.2 Methods

3.2.1 Patients

Adult patients attending the renal unit at Glasgow Royal Infirmary between 1992 and 2007 who had at least one UNa/24h measurement were identified using the electronic patient record. Urinary electrolytes were measured using flame photometry in a standard laboratory. Baseline drug therapy, renal function, proteinuria, weight, blood pressure and Charlson index of comorbidity were recorded. Advice regarding oral sodium intake was physician determined and patients were not routinely assessed by a dietician. Patients who were receiving RRT at the time of measurement, patients without a weight recording, patients without a 4v-MDRD eGFR recording at time of measurement and at least one further reading, and patients with a decline in eGFR >10ml/min/yr were excluded.

Fractional excretion of sodium = (Serum Cr * Urine Na) / (Serum Na * Urine Cr)%

3.2.2 Adequacy of collection

Oral sodium intake in the West of Scotland is high and patients with a UNa/24h less than 70mmol were presumed to have incomplete collections and excluded on this basis (n=278). Following this**,** measured creatinine clearance was compared with estimated creatinine clearance using the Cockroft and Gault formula ((Expected – Observed) / Expected)) x 100. A previous comparison showed that 94% of values estimated by this formula will fall within ± 35% of observed creatinine clearance(261). Using this analysis, 17% of collections fell out with the ± 35% range, with the majority (69%) underestimating, suggesting incomplete collections, which will underestimate the total urinary sodium for 24h. (Figure 3-1). Calculation of the UNa:Cr ratio and FENa is not dependent on the accuracy of collection.

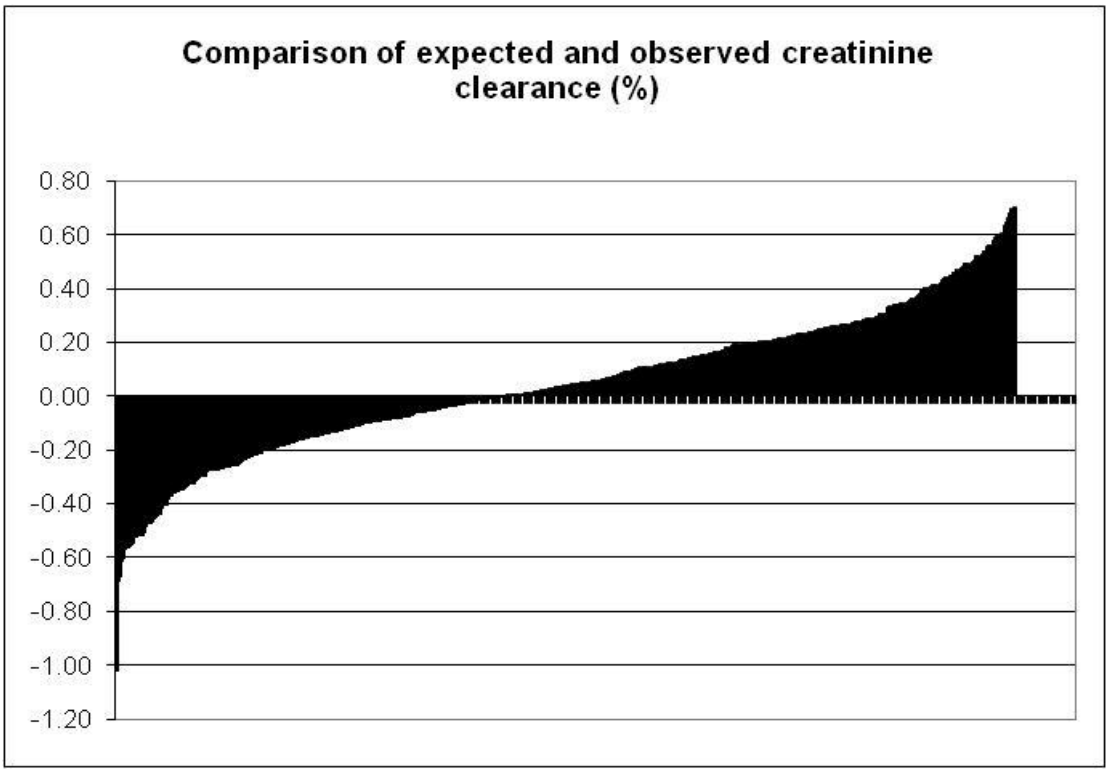


Figure 3-1: Urine collection adequacy.
Percentage derived from the expected creatinine clearance (measured using the Cockcroft and Gault formula) divided by the observed urinary creatinine clearance, multiplied by the expected %. ((E/O) X E)%.

3.2.3 Outcomes and analysis

Dates of starting renal replacement therapy (RRT) or death were recorded. Annual decline in eGFR was also calculated. Baseline demographics were compared using Student's T test, Mann Whitney U test, Chi Square test or one-way ANOVA as appropriate. Correlations between urinary sodium measures and other factors were ascertained using Spearman's or Pearson's correlation coefficients, depending on normality of the data.

Kaplan Meier survival analysis was performed based on time to RRT or death for tertiles of UNa and UNa:Cr. Binary logistic regression was performed to determine independent predictors of the requirement for RRT and multiple factors were then entered into a Cox survival model. Different measures of urinary sodium excretion were entered separately due to collinearity.

3.3 Results

3.3.1 Baseline demographics

Table 3-1 summarises baseline demographics for the whole cohort. Four hundred and eighty eight patients were included. The primary renal disease was recorded as renovascular disease (n=76), diabetic nephropathy (n=34), tubulointerstitial disease / polycystic kidney disease (n=57), glomerulonephritis (n=55) and others (n=16). The remainder were classified as CRF. One hundred and three patients (21.1%) required RRT for CRF at a median of 2.2 (0.8-4.9) years. 113 patients died (23.2%) during follow-up at a median of 3.8 (IQR 2.0-6.7) years. Censoring for death or RRT, median follow-up for the whole cohort was 8.6 (IQR 4.2-11.4) years. Overall mean slope decline in eGFR was -2.8 (4.0) ml/min/yr.

Variable	*Result*
Gender (% male)	50.4
Age (years)	51.8 ±16.7
Weight (Kg)	75.6±18.0
eGFR at baseline (ml/min/1.73m^2)	48.4 ± 25.6
SBP (mmHg)	139 ± 24
DBP (mmHg)	79 ± 13
ACR (mg/mmol)	10.0 (2.0-85.8)
USod (mmol/24h)	159.3 ± 63.7
UNa:Cr	16.6 ± 6.3
FENa (%)	1.44 (1.0-2.5)
UPot (mmol/24h)	55.5 ± 20.8
Diuretic therapy (%)	23.3
ACEi / ARB (%)	25.4
Deaths (n,%)	113 (23.2%)
RRT (n,%)	103 (21.1%)
Time to death (years)	3.8 (2.0-6.7)
Time to RRT (years)	2.2 (0.8-4.9)
eGFR loss (ml/min/1.73m2/yr)	-2.8 (4.0)

Table 3-1: Baseline demographics of cohort.
Mean ± SD or median (IQR).

3.3.2 Urinary sodium excretion in patients with CKD

Urinary sodium excretion is lower in patients with more advanced renal failure. As can be seen in Figure 3-2, the predominant reduction is in CKD stage 5, although there is a significant difference across all 5 groups (p=0.002).

Fractional excretion of sodium is higher in patients with reduced renal function. Figure 3-3 shows the scatterplot of log FENa versus log MDRD4 eGFR (R^2 = 0.79) i.e. renal function is the predominant determinant of fractional excretion of sodium. Figure 3-4 demonstrates a 3D boxplot of eGFR by tertile of UNa and tertile of FENa. It can be seen that those with a high FENa and low UNa have the lowest eGFR.

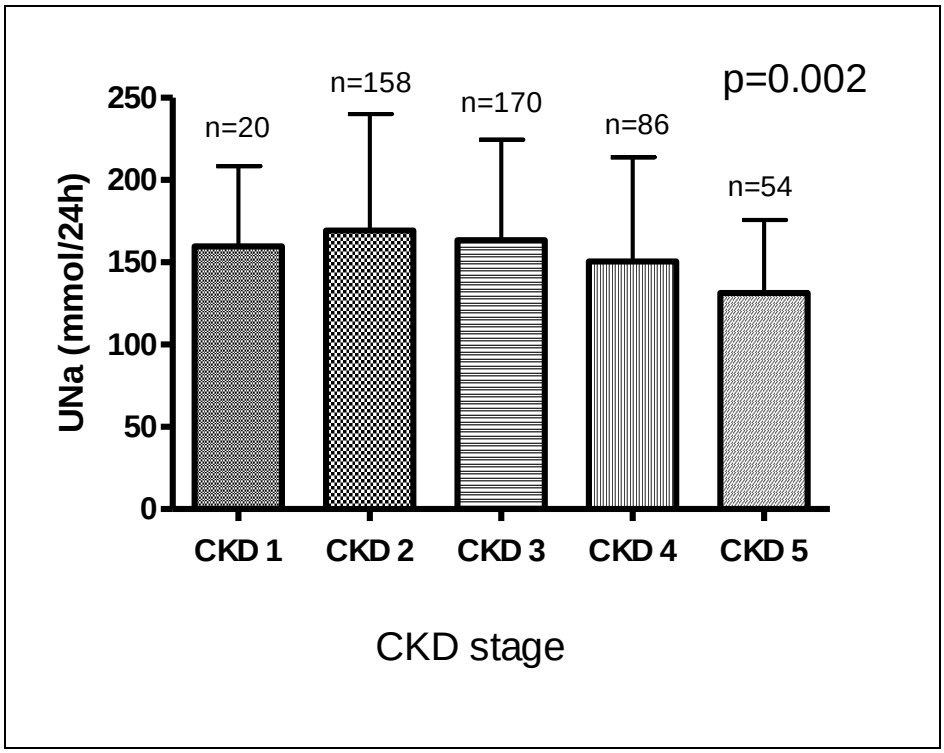


Figure 3-2: Bar chart of median urinary sodium excretion (mmol/24h) by CKD stage.
P=0.002 by Kruskall Wallis test.

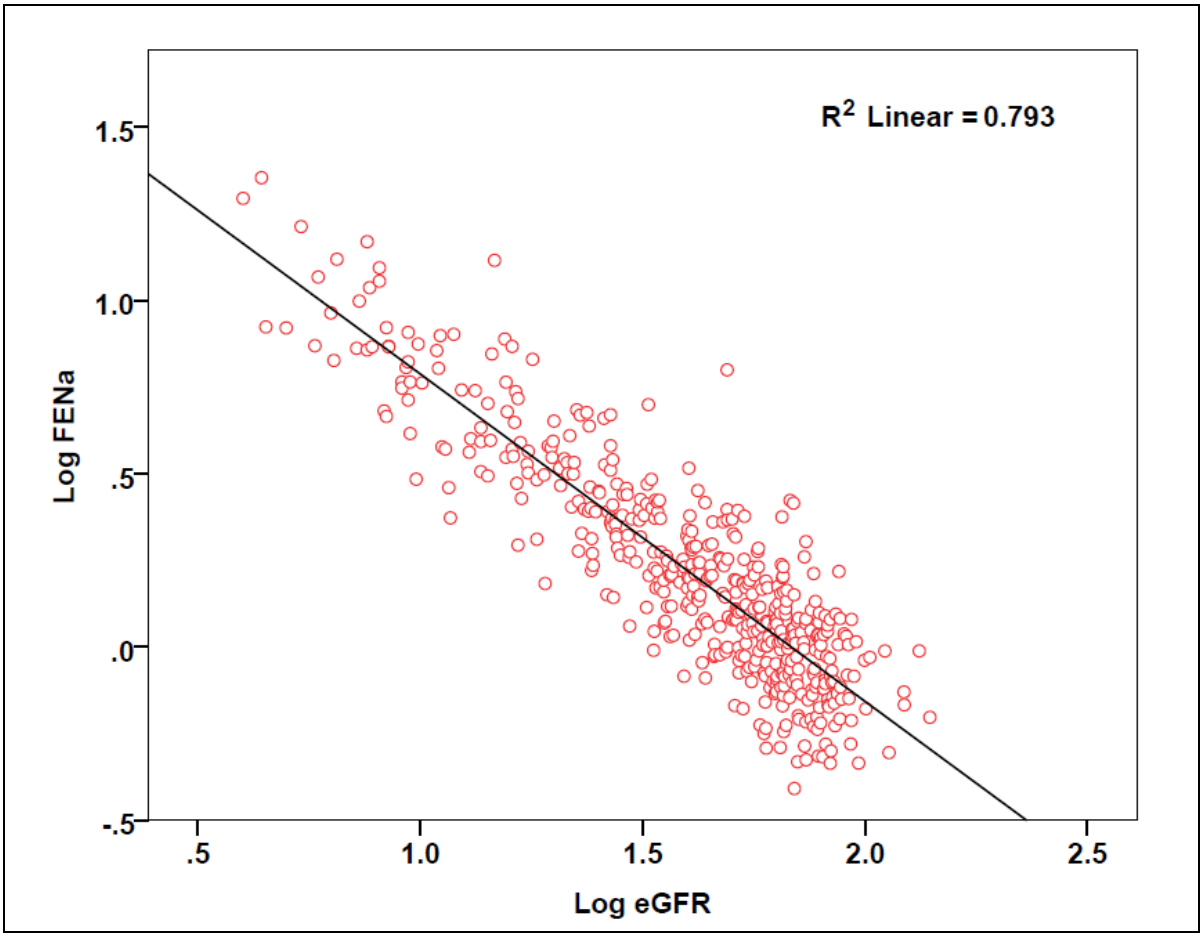


Figure 3-3: Log-log plot of FENa and eGFR.
Fitted linear regression line and R-square value derived from linear regression.

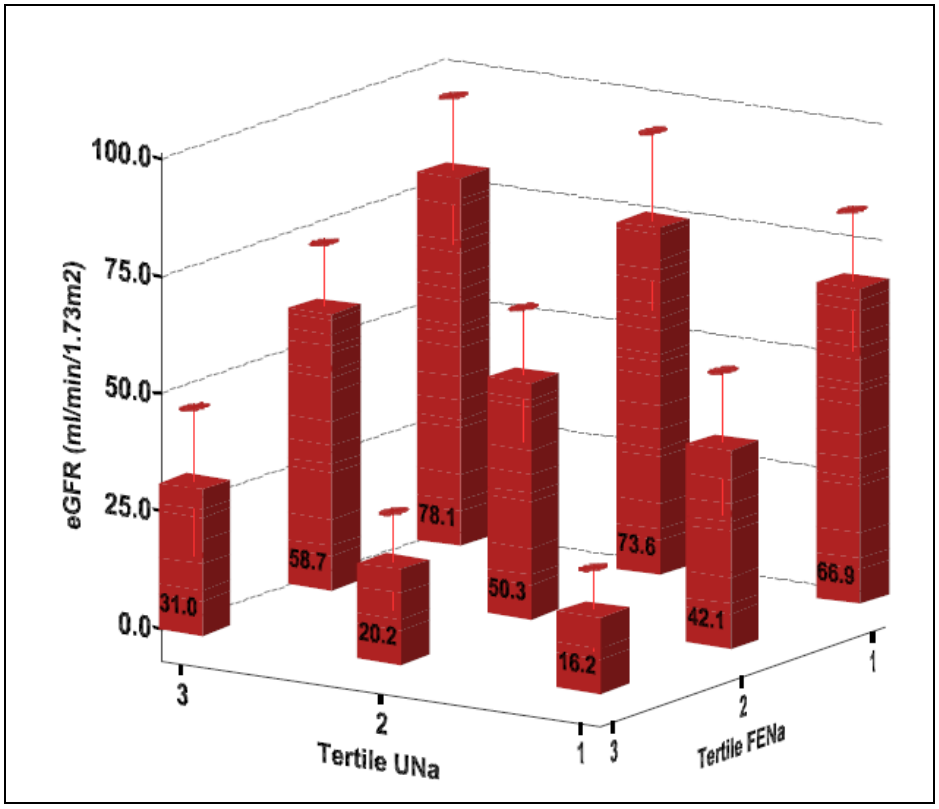


Figure 3-4: 3D boxplot of mean eGFR by tertile of UNa and tertile of FENa.
Error bars = 1SD. 1= Lowest tertile, 3=Highest tertile.

3.3.3 Correlations

To ascertain which factors correlated significantly with UNa, the following factors were entered into a correlation matrix: age, gender, weight, eGFR, diuretic usage, ACEi usage, blood pressure, proteinuria, RRT, death and eGFR loss over time.

3.3.3.1 UNa correlations

UNa correlated significantly with higher weight, higher eGFR, male gender and requirement for RRT. There was no significant correlation seen with blood pressure, age, death, diuretic therapy, inhibitors of the renin-angiotensin system or eGFR loss over time (Table 3-2).

Due to the close correlations between UNa and weight (r=0.379), weight and urinary creatinine (r=0.466) and UNa and urinary creatinine (r=0.543), the correlation matrix was repeated after adjusting UNa for weight in Kg and adjusting for urinary creatinine excretion. Adjusting for weight in Kg failed to remove the correlation between UNa and weight but adjusting for urinary creatinine did, therefore this measure was adopted for further analysis. There was no correlation between eGFR and weight.

3.3.3.2 UNa:Cr correlations

UNa:Cr correlated significantly with older age, male gender, higher SBP, lower eGFR, higher log ACR, diuretic usage, requirement for RRT and death. There was no significant correlation seen with DBP, weight, ACE usage or eGFR loss over time (Table 3-2).

	UNa/24h		*UNa/Kg/24h*		*UNa:Cr*	
Variable	**r**	**P**	**r**	**p**	**r**	**p**
DBP	0.11	0.017	**-0.293**	**<0.001**	0.062	0.176
SBP	0.1	0.031	-0.06	0.196	**0.191**	**<0.001**
eGFR	**0.151**	**0.001**	**0.183**	**<0.001**	**-0.226**	**<0.001**
Male gender	**0.296**	**<0.001**	**-0.248**	**<0.001**	**0.183**	**<0.001**
Age	-0.032	0.48	-0.044	0.342	**0.23**	**<0.001**
Weight	**0.379**	**<0.001**	**0.267**	**<0.001**	-0.071	0.116
LogACR	-0.105	0.033	**-0.14**	**0.004**	**0.18**	**<0.001**
RRT	**-0.115**	**0.01**	**-0.118**	**0.01**	**0.194**	**<0.001**
Death	-0.109	0.016	-0.68	0.138	**0.243**	**<0.001**
eGFR loss/yr	0.066	0.146	0.102	0.026	-0.059	0.192
Diuretic usage	-0.033	0.467	-0.003	0.944	**0.201**	**<0.001**
ACEi usage	0.072	0.113	0.046	0.321	0.115	0.011

Table 3-2: Correlation matrix between demographic variables and measures of urinary sodium excretion.

Correlations – Pearson's or Spearman's as appropriate. P<0.01 = significant (highlighted in bold and cell coloured yellow).

3.3.4 Urinary sodium and risk of requirement for RRT or death

To ascertain whether urinary sodium excretion was associated with an increased risk of death or requiring RRT, patients were separated into tertiles of UNa and UNa:Cr and Kaplan Meier analysis was performed.

Table 3-3 and 3-4 demonstrates the demographics of patients in each tertile. Those in the highest tertile of UNa had the highest eGFR and highest weight. Those in the highest tertile of UNa:Cr had the highest urinary sodium and lowest eGFR, likely reflecting reduced urinary creatinine excretion.

	All patients N=488	***T1 UNa N=163***	***T2 UNa N=162***	***T3 UNa N=163***	***P***
eGFR (ml/min/1.73m2)	48.5 (25.6)	43.1 (25.5)	51.7 (27.1)	51.0 (23.7)	**0.004**
Age (y)	51.6 (16.7)	53.4 (16.9)	49.0 (16.9)	52.2 (16.1)	NS
% male	50.4	33.3	50.0	67.7	**<0.001**
SBP (mmHg)	139.0 (23.0)	136.5 (24.7)	139.0 (22.2)	141.3 (21.8)	NS
LogACR	1.1 (0.9)	1.2 (0.9)	1.1 (0.9)	1.1 (0.9)	NS
Weight (Kg)	75.6 (18.0)	68.8 (15.2)	74.2 (16.3)	83.7 (19.0)	**<0.001**
Charlson co-morbidity score	2.3 (1.6)	2.4 (1.5)	2.2 (1.6)	2.2 (1.7)	NS
UNa (mmol/24h)	159.4 (63.8)	100.3 (15.7)	147.9 (15.2)	228.8 (56.7)	**<0.001**
UNa:Cr	16.6 (6.3)	14.4 (5.3)	15.5 (4.8)	19.9 (7.1)	**<0.001**
UCr (mmol/24h)	10.2 (3.7)	7.8 (2.8)	10.3 (2.9)	12.5 (3.8)	**<0.001**
U Potassium (mmol/24h)	55.4 (20.8)	42.1 (16.0)	55.8 (18.0)	66.8 (20.0)	**<0.001**
eGFR loss (ml/min/yr)	-2.8 (2.8)	-2.8 (3.3)	-2.7 (4.2)	-2.8 (4.0)	NS
RRT (n, %)	102, 20.9	40 (24.7)	32 (19.8)	31 (18.9)	NS
Deaths (n, %)	113, 23.2	49 (30.2)	28 (17.3)	36 (22.0)	**0.02**

Table 3-3: Demographics of patients in each tertile of UNa.

T1 = Tertile 1; T2 = Tertile 2; T3 = Tertile 3. Comparisons by one-way ANOVA, Chi-square or Kruskall-Wallis test as appropriate.

Variable	**All patients N=488**	***T1 UNa:Cr N=163***	***T2 UNa:Cr N=162***	***T3 UNa:Cr N=163***	
eGFR (ml/min/1.73m^2)	48.5 (25.6)	55.4 (23.9)	48.3 (26.0)	41.9 (25.1)	**<0.001**
Age (y)	51.6 (16.7)	46.5 (14.8)	51.7 (17.2)	56.7 (16.6)	**<0.001**
% male	50.4	60.9	56.3	34.2	**<0.001**
SBP (mmHg)	139.0 (23.0)	134.2 (21.8)	139.4 (22.5)	143.6 (23.8)	**0.001**
LogACR	1.13 (0.89)	0.93 (0.85)	1.15 (0.84)	1.32 (1.0)	**0.001**
Weight (Kg)	75.6 (18.0)	77.1 (17.5)	76.4 (19.1)	73.3 (75.6)	NS
Charlson co-morbidity score	2.3 (1.6)	1.9 (1.3)	2.3 (1.7)	2.5 (1.7)	**0.008**
UNa (mmol/24h)	159.4 (63.8)	128.6 (37.7)	160.0 (55.5)	189.7 (76.8)	**<0.001**
U Cr (mmol/24h)	10.2 (3.7)	12.1 (3.5)	10.3 (3.5)	8.2 (3.2)	**<0.001**
U Pot (mmol/24h)	55.4 (20.8)	55.2 (19.7)	56.7 (19.3)	54.4 (22.9)	NS
eGFR loss (ml/min/yr)	-2.8 (2.8)	-2.5 (2.7)	-2.5 (2.8)	-2.9 (2.8)	NS
Dead (n, %)	113, 23.2	20, 12.3	33, 20.4	60, 36.8	**<0.001**
RRT (n, %)	102, 20.9	22, 13.5	37, 22.8	43, 26.4	**0.004**

Table 3-4: Demographics of patients in each tertile of UNa:Cr.
T1 = Tertile 1; T2 = Tertile 2; T3 = Tertile 3. Comparison via One-way ANOVA or Chi square as appropriate.

3.3.5 Kaplan Meier survival analysis of time to RRT or death

3.3.5.1 UNa

Kaplan Meier analysis of time to RRT by tertile of UNa was performed and revealed no significant difference in risk between tertiles. With regards to risk of dying, those in T1 (lowest tertile) were significantly more likely to die than those in the T2 ($p=0.006$) and there was a trend to an increased risk in T2 compared with those in the T3 (0.093).

3.3.5.2 UNa:Cr

Those in the T3 (the highest tertile) of UNa:Cr were significantly more likely to require RRT ($p=0.01$) (Figure 3-5) or die ($p<0.001$) (Figure 3-6) compared with those in T1 (the lowest tertile). The relationships were unaltered when looking at gender.

Therefore, those with a high UNa:Cr had a worse outcome with regards to risk of death or requiring RRT than those with a low UNa:Cr. Conversely, tertile of UNa had no effect on risk of requiring RRT but those in the lowest tertile of UNa were more likely to die.

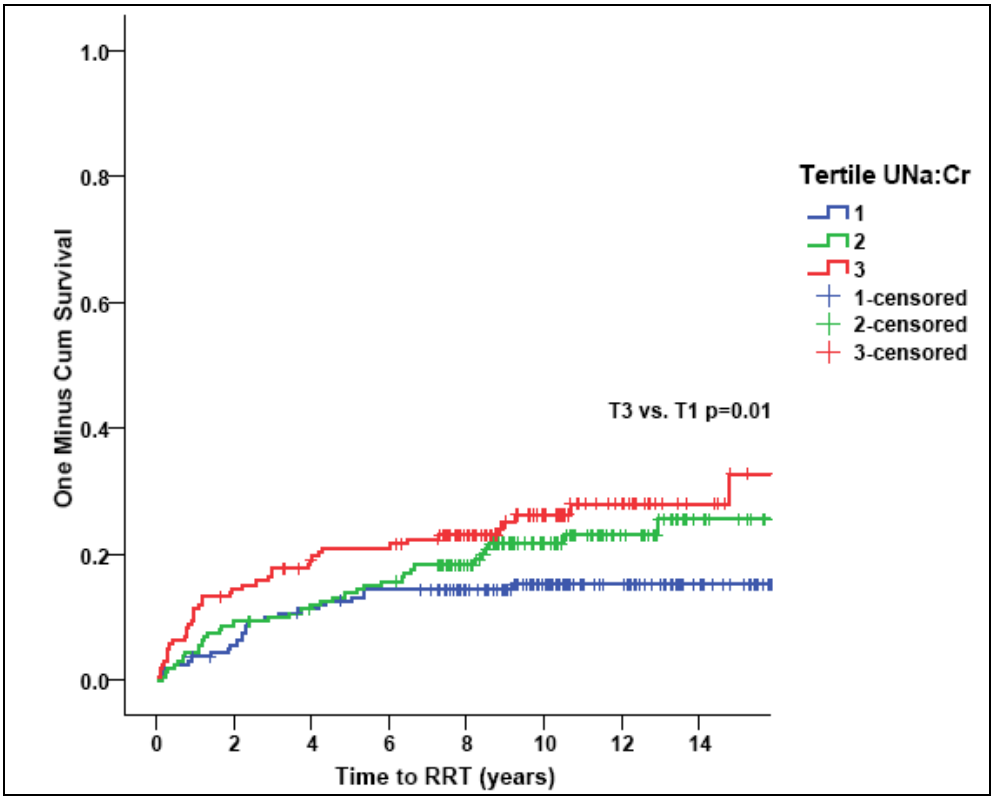


Figure 3-5: 1-survival plot of time to RRT by tertile of UNa:Cr.
Comparison by log-rank test with estimate of significance.

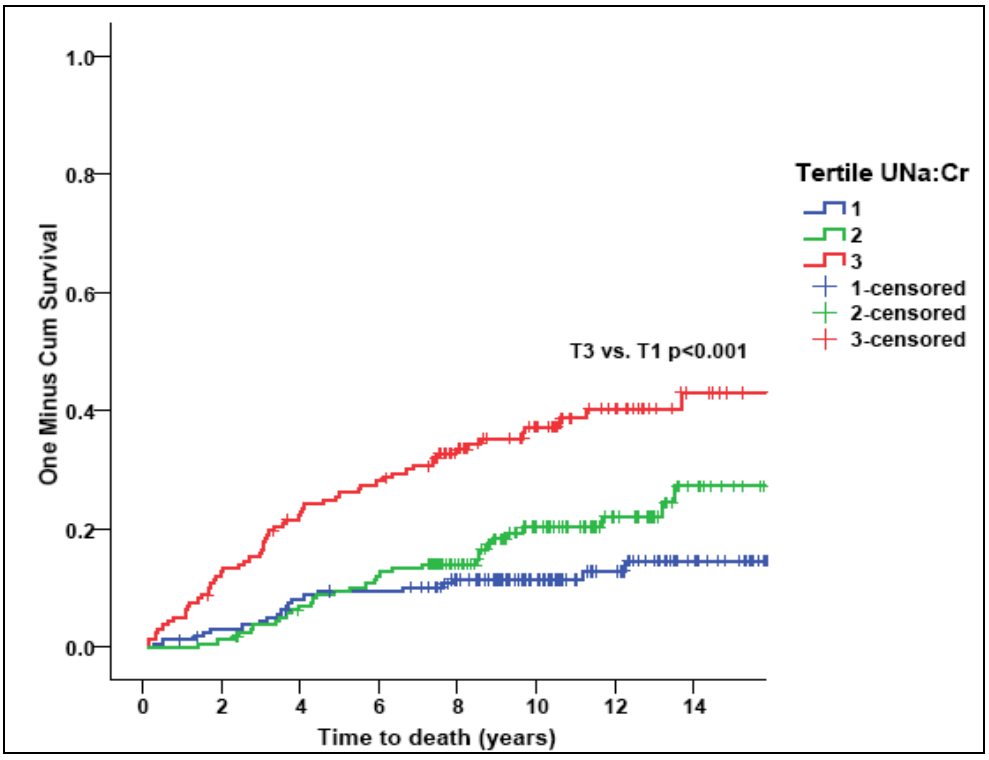


Figure 3-6: 1-survival plot of time to death by tertile of UNa:Cr.
Comparison by log rank test and estimate of significance.

3.3.6 Binary logistic regression model of factors predictive of death

On univariate analysis UNa:Cr (ExpB 1.09 (1.05-1.13) p<0.001), UNa (0.995 (0.992-0.999, p=0.02), age (ExpB 1.08 (1.06-1.10) p<0.001), SBP (ExpB 1.03 (1.02-1.04) p<0.001), eGFR (ExpB 0.95 (0.93-0.96) p<0.001) and logACR (ExpB 2.87 (2.1-3.9) p<0.001) were significant predictors of risk of death.

3.3.7 Cox survival analysis of risk of dying

When all significant univariate predictors of death were entered into a Cox survival model, UNa:Cr remained a significant independent predictor of death – the higher the Na:Cr, the higher the risk (Table 3-5, Figure 3-6) along with eGFR, age and logACR. UNa was not a significant independent predictor of death.

Variable	***Exp(B)***	***95% CI***	***P***
eGFR (ml/min)	0.971	0.96-0.98	**<0.001**
Age (years)	1.054	1.03-1.08	**<0.001**
Log ACR	1.596	1.171-2.174	**0.003**
UNa:Cr	1.035	1.0-1.07	**0.042**
SBP (mmHg)	1.005	1.0-1.01	0.35

Table 3-5: Cox survival model for risk of dying.
Chi square value for model = 131.0.

3.3.8 Binary logistic regression model of factors predictive of requirement for RRT

To clarify whether urinary sodium excretion was an independent predictor of outcome, binary logistic regression and Cox survival modelling was undertaken.

On univariate analysis, UNa (ExpB = 0.995 (0.991-0.999) p=0.01), UNa:Cr (Exp B 1.071 (1.036-1.108) p<0.001), baseline eGFR (ExpB 0.903 (0.89-0.92) p<0.001), log ACR (ExpB 5.017 (3.4-7.4) p<0.001), age (ExpB 1.024 (1.01-1.04) p=0.001) and SBP (ExpB 1.021 (1.011-1.031) p<0.001) were all significant predictors of requirement for RRT. Gender was not a significant independent predictor.

3.3.9 Cox survival analysis of risk of requiring RRT

Significant univariate predictors of requirement for RRT were entered together into a Cox survival model to ascertain which factors were significant independent predictors. UNa and UNa:Cr were entered separately due to high collinearity. Neither UNa:Cr nor UNa were

significant independent predictors of time to RRT when entered separately into a Cox survival model with age, SBP, logACR and eGFR. eGFR and logACR were significant independent predictors of risk of requiring RRT.

Variable	*Exp(B)*	*95% CI*	*P*
eGFR (ml/min/1.73m^2)	0.91	0.892-0.928	**<0.001**
Log ACR	2.392	1.609-3.557	**<0.001**
Na:Cr	1.002	0.965-1.041	0.911
UNa (mmol/24h)	0.999	0.995-1.003	0.703
Age (years)	0.989	0.974-1.005	0.181
SBP (mmHg)	1.004	0.994-1.013	0.452

Table 3-6: Cox survival model for risk of requiring RRT.
Chi square for model =246.4.

3.3.10 Urinary sodium, blood pressure and proteinuria

On linear regression analysis, UNa:Cr was a significant independent predictor of mean arterial pressure (p=0.001). The relationship persisted when patients prescribed diuretics were excluded. Similarly, UNa was a significant independent predictor of MAP p=0.005). Both measures of urinary sodium were also significant independent predictors of logACR on linear regression (UNa p=0.03, UNa:Cr p=0.001) after adjusting for blood pressure and renal function.

3.3.11 Urinary potassium as a predictor of outcome

UK/24h correlated significantly with baseline renal function (r=0.235, p<0.001), risk of death (r= -0.156, p=0.003) or RRT (r= -0.16, p=0.011). UK/24h was a significant univariate predictor of death on binary logistic regression (ExpB 0.981 (0.97-0.99) p=0.004) and RRT (Exp B 0.98 (0.97-0.99) p=0.012), but not independent of renal function. UK:Cr did not correlate with risk of RRT or death. There was no correlation between UK:Cr or UK/24h and SBP or logACR.

3.4 Discussion

This study has demonstrated the following key findings:

- Urinary sodium excretion is lower in patients with lower eGFR
- Fractional excretion of sodium is higher in patients with a lower eGFR
- A high urinary sodium, adjusted for urinary creatinine excretion, is independently associated with an increased risk of death in patients with CKD
- Urinary sodium excretion is associated with requirement for RRT, but not independently of eGFR or proteinuria excretion
- No measure of urinary sodium is associated with rate of decline in renal function

3.4.1 Sodium excretion and eGFR

The kidney adapts to reduction in nephron mass by altering its handling of sodium and increasing the fractional excretion of sodium. As the overall glomerular filtration rate declines with falling nephron mass, there is an increase in single nephron GFR, decreased proximal tubular sodium reabsorption and altered capacity of the distal tubule to reabsorb sodium(262), increasing the fractional excretion of sodium. Through this mechanism, the kidneys maintain the ability to adjust to changes in sodium, thus maintaining equilibrium between sodium intake and excretion.

Despite this, in this study it was shown that urinary sodium excretion is lower in patients with a lower eGFR, most markedly in CKD 5. This may be due to a reduced dietary intake, perhaps due to loss of appetite. Alternatively, despite the increase in the fractional excretion of sodium, perhaps the failing kidneys are unable to fully excrete sodium appropriately. However, even if sodium was stored in the recently proposed non-osmotically active form(263) the accumulation of vast sodium stores over a number of years would likely lead to storage sites being overwhelmed, water retention and catastrophic consequences.

3.4.2 Reporting of urinary sodium excretion rates

24h urinary sodium excretion is a good marker of dietary sodium intake, even in patients with reduced eGFR, where 90% of ingested sodium is excreted in the urine under thermoconstant conditions (55). In this study, urinary sodium was adjusted for urinary creatinine excretion, which likely had two purposes. It will adjust for muscle mass, but may also take into account reduced GFR or reduced tubular secretion of creatinine, as a reflection of renal failure. It also removes uncertainty about the completeness of a 24h urine collection, although using a 24h sample to measure the ratio ensures it will accurately represent daily intake.

3.4.3 Urinary sodium and risk of requirement for RRT or death

Assessing whether urinary sodium excretion, the marker of dietary sodium intake, is associated with risk of renal progression and requirement for RRT will be confounded by baseline eGFR. To control for this, if one assumes that progression of renal disease is predominantly linear and unaffected by GFR, the absence of any association of urinary sodium measures with annual slope decline in eGFR suggests that sodium is not primarily involved in determining rate of renal decline. This is supported by the finding that urinary sodium excretion was not an independent predictor of requirement for renal replacement therapy, after adjusting for eGFR and urinary ACR, whichever measure was used.

After adjusting urinary sodium for urinary creatinine excretion, those in the highest tertile of UNa:Cr had the highest urinary sodium excretion and the highest risk of death, even after adjusting for age, renal function, blood pressure and proteinuria. Those in the highest tertile of UNa:Cr also had the lowest urinary creatinine suggesting that a high dietary sodium intake should be viewed in proportion to muscle mass and body weight. This is a novel finding in patients with CKD.

Dietary sodium intake was high in this cohort, however similar to a male cohort from Italy(264). Current recommendations are for a daily salt intake of not more than 6g/day (approximately 100mmol of sodium)(55), which our patients clearly exceed by around 60%. This is despite a likelihood of 15% of patients providing an incomplete urinary collection.

3.4.4 Potential pathogenic mechanisms of high sodium intake

The potential pathogenic mechanisms explaining sodium mediated damage are multiple. Haemodynamic effects mediated via volume retention, resulting in increased shear stress, endothelial dysfunction, elevated preload, vascular stiffness and elevated afterload and left ventricular hypertrophy are plausible. Non-haemodynamic factors such as oxidative stress via superoxide production(67) and inflammatory mediators have been widely studied in rat models (68) (265;266). Normotensive and hypertensive rats fed a high salt diet developed vascular, glomerular and interstitial fibrosis(73), suggesting a directly nephrotoxic effect of dietary sodium.

3.4.5 Population evidence of negative effects of high dietary sodium intake

Damaging effects of dietary sodium on end-organs have been shown in a variety of populations. In observational studies, a study of normotensive and never treated hypertensives (59) demonstrated that patients with a high dietary sodium intake exhibited increased left ventricular mass and albuminuria compared with those in the lowest quintile, which they hypothesised to be due to an amplification of the effect of hypertension. The Framingham Offspring Study, using a spot urinary sodium sample normalised to creatinine, demonstrated that each standard deviation increment in log urine sodium index to be associated with a 22% increase in urinary ACR, similar to my findings and also replicated by others(60;61). The Scottish Heart Health Study, looking at 11629 healthy middle aged individuals, with median urinary sodium excretion of 228 mmol/d, showed that urinary sodium excretion did not predict coronary heart disease in men but did in women, whilst urinary potassium excretion was protective(267).

Tempering the findings of sodium reduction being associated with a reduction in cardiovascular events are data from hypertensive patients demonstrating an inverse relationship between urinary sodium values and risk of cardiovascular morbidity and mortality(268). It was postulated by these authors that low sodium intake results in increased renin-angiotensin system activation and resultant detrimental effects. A recent Cochrane review failed to conclude that reducing dietary sodium intake was convincingly associated with a reduction in cardiovascular events(269). Furthermore, a recent large prospective study in a Flemish population without cardiovascular disease found low urinary sodium excretion to be associated with an increased cardiovascular risk(270). Alderman has proposed a J-shaped effect, that when patients dietary sodium intake is particularly low or particularly high they are at increased cardiovascular risk(271).

Similar findings were seen in the NHANES I cohort, where estimates of dietary sodium intake showed an inverse relationship with mortality(272). There were however limitations in the study designs of these trials(273). It is difficult to directly compare these cohorts with the patients in this study as my data relate to patients with impaired renal function and alternative physiological explanations may exist for urinary sodium excretion.

The role of dietary potassium has largely been neglected despite good evidence that potassium intake is of great significance to vascular and endothelial health(64), and in INTERSALT the urinary potassium:sodium ratio had a significant inverse relationship with blood pressure, stronger than that of sodium or potassium excretion alone(54). In this study no evidence was found to support an independent protective effect of urinary potassium excretion in patients with CKD.

3.4.6 Evidence for a negative effect of dietary sodium in patients with CKD

In patients with renal disease, no studies have shown a high dietary sodium intake to be associated with the risk of requiring renal replacement therapy or death. A small cohort study from Italy(65) addressed the impact of sodium intake on progression of renal disease. They subdivided their group into those with a urinary sodium excretion of <100meq/day or >200meq/day and found the low sodium group to have a lower creatinine clearance at baseline, similar to our cohort, but a slower loss in renal function, which differs from our cohort, where no difference in rate of renal decline was noted in different groups.

Dietary sodium intake has been shown to reduce proteinuria excretion, independent of a reduction in blood pressure, over 6 weeks (66). In patients with end stage renal failure treated with peritoneal dialysis, a low dietary sodium intake independently predicted increased risk for overall and cardiovascular death and was not explained by adjusting for deficient protein and energy intake(274).

It should be emphasised that the anti-proteinuric response to inhibitors of the renin-angiotensin system, the mainstay of drug therapy for renal protection, is augmented by a low sodium diet(275), presumably due to effects on volume status, providing a powerful argument for salt restriction in patients with CKD.

3.4.7 Limitations

This was a retrospective cohort analysis, although data were prospectively recorded, using real world patients prescribed a number of different medications; however by necessity most such studies are observational. An interventional approach is required to be certain of risks and benefits. Variations in diet during follow-up are not accounted for. An assessment of nutritional status would have been useful in this cohort.

3.5 Conclusions

As renal function declines, the kidneys compensate by increasing fractional excretion of sodium, but total urinary sodium excretion is lower in CKD5. Urinary sodium is closely related to weight and adjustment for urinary creatinine addresses this issue.

A high urinary sodium:creatinine ratio, reflective of a proportionally high dietary sodium intake, in patients with chronic kidney disease, is associated with a significantly and independently increased risk of death. This is a novel finding and the first to confirm relationship between sodium intake and mortality in CKD. No measure of urinary sodium excretion was independently associated with decline in renal function or requirement for RRT.

4 Chapter 4 - Mineralocorticoids and the renin-angiotensin system in chronic kidney disease and essential hypertension; Determinants, impact of therapy, effects on end-organs and interaction with sodium.

4.1 Introduction

Patients with primary hyperaldosteronism, or hypertension in the context of an elevated aldosterone to renin ratio, suffer more adverse cardiovascular events than patients with essential hypertension(276). Animal models have shown that high aldosterone or deoxycorticosterone levels, together with a high sodium intake, affect the heart and kidney. Biventricular cardiac hypertrophy and fibrosis, renal glomerulosclerosis and tubular atrophy are seen (141). A number of clinical trials have demonstrated the cardiovascular benefits of mineralocorticoid receptor antagonists in patients with heart failure(125) or post-myocardial infarction(126) and anti-proteinuric effects in patients with CKD(119). These findings are discussed in detail in section 1.5.5.

Despite significant laboratory and interventional trial evidence of the benefits of MR blockade, there remains a paucity of data regarding the physiological control and actions of mineralocorticoids in CKD. Older studies assessing plasma aldosterone levels in patients with CKD suggest that aldosterone levels rise as renal function falls(277). However, the value of single measurements of plasma aldosterone levels is limited by the impact of posture, episodic secretion of the hormone and diurnal variation, although less so than for cortisol.

Measurement of urinary steroid metabolites over 24h is more representative of aldosterone status as it eliminates these variations, but it has not been widely adopted in studies of renal disease (See section 1.4.9).

The aims of this study were therefore as follows:

1. To compare levels of aldosterone, deoxycorticosterone and cortisol in patients with CKD with those in patients with EH, as a control group. Are levels higher in patients with CKD for the same level of blood pressure?

2. Do patients with diabetes mellitus have higher steroid levels than other patient groups?

3. Are determinants of mineralocorticoid excretion rates (reflecting regulation of synthesis), similar in patients with CKD and patients with EH?

4. Are steroid levels associated with end-organ damage as exemplified by left ventricular mass, proteinuria, pulse wave velocity and endothelial function?

5. Does the level of sodium intake modulate the damaging effects of steroids?

4.2 Methods

Patients were recruited and assessed according to the protocol described in chapter 2. Statistical analysis was performed using SPSS (Illinois) version 15.0.

4.3 Results

4.3.1 Patient Demographics

Table 4-1 describes the baseline demographics of the cohort, stratified by group.

Patients with EH and CKD were well matched with no significant differences in gender, age, weight or LV mass. EH patients had a significantly higher DBP and significantly lower PWV. Within the CKD cohort, patients with IgAN were significantly younger and had significantly lower SBP. Diabetic patients had a significantly higher PWV and LVMI.

Fifteen patients agreed to undergo 24h blood pressure monitoring. There was close correlation between the clinic blood pressure reading average and the daytime average (SBP $p<0.001$, DBP $p<0.001$) and the 24h blood pressure average (SBP $p<0.001$, DBP $p<0.001$).

Biochemical differences between the EH and CKD cohorts are as would be expected (Tables 4-2 & 4-3). Renal patients had significantly lower eGFR levels, had higher concentrations of serum urate, CRP, potassium, serum phosphate and PTH and lower concentrations of vitamin D, serum albumin and haemoglobin. Serum sodium was not significantly different between groups, but serum potassium was significantly lower in the

EH group. Renal patients had higher levels of proteinuria and significantly lower levels of urinary potassium excretion rates. Within the renal cohort, patients with membranous nephropathy had better preserved renal function, lower levels of PRC and higher levels of serum cholesterol. Patients with diabetic nephropathy had poorer renal function and lower haemoglobin levels.

Variable	EH controls N=30	CKD patients N=70	p	DMN N=34	IgAN N=27	MGN N=9	p
Age (y)	55.4 (9.2)	58.2 (12.8)	NS ∞	**61.8 (13.0)**	**52.9 (12.1)**	**60.5 (9.2)**	**0.019** Δ
% Male	83.3	75.7	NS δ	76.5	77.8	66.7	NS δ
Smoker (%) **-Current** **-Ex** **-Never**	 10 26.7 63.3	 20.0 32.9 47.1	 NS δ	 14.7 41.2 41.2	 18.5 22.2 59.3	 33.3 33.3 33.3	 NS δ
Weight (Kg)	88.4 (15.2)	85.6 (19.7)	NS ∞	88.0 (16.6)	85.7 (25.1)	76.2 (6.6)	NS Δ
BMI (Kg/m2)	29.3 (4.8)	29.3 (5.8)	NS ∞	30.7 (5.7)	28.4 (6.1)	26.8 (4.9)	NS Δ
Waist circumference (cm)	100.5 (12.1)	99.9 (13.5)	NS ∞	104.5 (12.0)	96.7 (15.3)	92.7 (7.3)	0.021 Δ
SBP (mmHg)	151.5 (19.7)	147.2 (23.0)	NS ∞	**156.8 (21.3)**	**135.6 (17.9)**	**145.4 (28.4)**	**0.001** Δ
DBP (mmHg)	**93.4 (11.4)**	**82.1 (12.3)**	**<0.001** ∞	80.0 (13.4)	85.6 (10.0)	83.0 (13.7)	NS Δ
MAP (mmHg)	112.8 (13.3)	103.8 (14.0)	NS ∞	105.6 (14.9)	101.6 (11.7)	103.8 (17.6)	NS Δ
PP (mmHg)	**58.1 (13.4)**	**65.0 (19.0)**	**0.004** ∞	**76.8 (14.9)**	**51.0 (13.1)**	**62.4 (19.5)**	**<0.001** Δ
HR (bpm)	70.2 (12.5)	72.3 (13.4)	NS ∞	74.7 (14.4)	70.9 (12.6)	67.4 (10.9)	NS Δ
ECG abnormality (%)	16.7	35.3	0.049 ∞	45.5	35.3	0	NS Δ
AIx	26.4 (11.6)	23.7 (10.3)	NS ∞	24.6 (10.7)	21.1 (9.7)	27.9 (10.1)	NS Δ
AIx@75bpm	24.9 (10.8)	22.1 (10.1)	NS ∞	23.7 (10.0)	19.1 (10.4)	24.7 (8.8)	NS Δ
PWV (m/s)	**7.9 (6.7-9.1)**	**9.4 (7.3-13.5)**	**0.006** ∞	**11.8 (9.3-15.3)**	**7.0 (5.8-9.4)**	**7.7 (6.3-10.3)**	**<0.001** Δ
RHI	1.9 (1.7-2.4)	1.8 (1.5-2.4)	NS ∞	1.6 (1.4-2.2)	2.0 (1.6-2.5)	1.8 (1.5-2.7)	NS Δ
EF (%)	68.1 (10.5)	68.9 (10.9)	NS ∞	71.0 (7.7)	67.1 (12.6)	67.9 (13.6)	NS Δ
cEDV (ml/m^2)	67.1 (15.6)	60.3 (16.1)	NS ∞	57.8 (12.0)	62.4 (11.8)	61.7 (31.6)	NS Δ
cESV (ml/m^2)	21.8 (19.9)	19.9 (10.3)	NS ∞	16.9 (6.2)	21.3 (9.4)	24.5 (19.1)	NS Δ
MC mass ED (g)	175.9 (53.5)	170.3 (49.8)	NS ∞	**193.1 (50.0)**	**151.6 (41.7)**	**160.4 (50.5)**	**0.01** Δ
LVMI (g/m^2)	87.1 (22.2)	84.7 (20.4)	NS ∞	**94.1 (19.9)**	**75.4 (15.0)**	**84.3 (25.1)**	**0.004** Δ

Table 4-1: Baseline demographics stratified by group.
Data presented as mean (SD) or median (IQR). Comparisons made using t-test (∞) or one-way ANOVA (Δ) for normally distributed data and Mann Whitney U-test (ε) or Kruskall-Wallis test (γ) for non-parametric analysis. Categorical variables were compared using the Chi Square test (δ). P<0.01 = significant (highlighted in bold).

Variable	EH controls N=30	CKD patients N=70	p	DMN N=34	IgAN N=27	MGN N=9	p
MDRD4 (ml/min/1.73m^2)	**92.1 (18.5)**	**40.3 (24.2)**	**<0.001 ∞**	**32.4 (17.1)**	**41.1 (23.5)**	**64.3 (33.8)**	**0.001 Δ**
MDRD6 (ml/min/1.73m^2)	**90.4 (18.2)**	**38.6 (24.1)**	**<0.001 ∞**	**30.8 (17.9)**	**41.3 (24.1)**	**60.1 (32.0)**	**0.003 Δ**
C&G (ml/min/1.73m^2)	**113.8 (89.2-143.8)**	**45.3 (30.4-65.0)**	**<0.001 ε**	38.8 (27.7-53.2)	49.9 (29.3-88.1)	59.9 (44.9-100.5)	NS γ
Cr (umol/l)	**76 (66-88)**	**176 (129-258)**	**<0.001 ε**	**222 (155-268)**	**175 (113-261)**	**107 (71-153)**	**0.007 γ**
UCrCl	**129.9 (102.0-166.1)**	**43.3 (29.5-66.4)**	**<0.001 ε**	**35.5 (28.2-53.9)**	**46.7 (27.6-97.9)**	**62.7 (56.9-99.7)**	**0.01 γ**
Total Chol (mmol/l)	4.8 (0.9)	4.5 (1.2)	NS ∞	**4.0 (0.9)**	**4.6 (1.2)**	**5.8 (1.5)**	**<0.001 Δ**
HDL (mmol/l)	1.3 (0.4)	1.2 (0.5)	NS∞	1.13 (0.4)	1.3 (0.5)	1.4 (0.5)	NS Δ
LDL (mmol/l)	2.9 (0.8)	2.4 (0.9)	NS∞	2.0 (0.6)	2.7 (1.0)	3.2 (0.4)	NS Δ
Chol:HDL	3.9 (1.0)	4.0 (1.3)	NS∞	3.8 (1.3)	3.9 (1.1)	4.6 (1.7)	NS Δ
TG (mmol/l)	1.5 (1.2-2.2)	1.5 (1.1-2.2)	NS∞	1.4 (1.0-2.5)	1.6 (1.2-2.0)	1.1 (1.0-3.0)	NS γ
Urate (mmol/l)	**0.34 (0.09)**	**0.45 (0.13)**	**<0.001 ∞**	0.47 (0.13)	0.45 (0.13)	0.41 (0.1)	NS Δ
Sodium (mmol/l)	139 (2)	139 (3)	NS ∞	139 (3)	139 (3)	140 (2)	NS Δ
Potassium (mmol/l)	**3.8 (0.3)**	**4.6 (0.6)**	**<0.001 ∞**	4.7 (0.5)	4.5 (0.5)	4.5 (0.8)	NS Δ
CRP (mg/l)	**1.5 (0.9-3.6)**	**2.9 (1.5-6.2)**	**0.01 ε**	3.3 (1.7-6.5)	2.4 (1.1-4.3)	4.6 (1.3-9.1)	NS γ
Albumin (g/l)	**39.7 (3.3)**	**36.9 (3.9)**	**<0.001 ∞**	35.9 (4.4)	38.6 (2.9)	35.5 (2.4)	NS Δ
PO_4 (mmol/l)	**1.0 (0.23)**	**1.20 (0.23)**	**<0.001 ∞**	1.21 (0.19)	1.18 (0.28)	1.18 (0.20)	NS Δ
AdCal (mmol/l)	2.40 (0.07)	2.38 (0.10)	NS ∞	2.38 (0.08)	2.37 (0.12)	2.41 (0.10)	NS Δ
CaPO4	**2.39 (0.60)**	**2.80 (0.63)**	**0.003 ∞**	2.79 (0.68)	2.79 (0.61)	2.85 (0.54)	NS Δ
PTH (pmol/l)	**5.8 (4.8-6.7)**	**11.5 (6.0-20.9)**	**<0.001 ε**	14.6 (7.4-23.6)	9.0 (6.4-18.1)	6.5 (4.9-11.2)	NS γ
Vit D (ng/ml)	**19.5 (13.8-26.3)**	**13.0 (5.0-19.3)**	**0.005 ε**	12.0 (2.5-17.3)	11.0 (7-19)	18 (13-28)	NS γ
Hb (g/dl)	**14.6 (1.3)**	**12.3 (1.8)**	**<0.001 ∞**	**11.5 (1.6)**	**13.2 (1.6)**	**12.6 (1.4)**	**0.001 Δ**
PAC (pmol/l)	236.5 (163.2-331.3)	254.5 (162.3-442.5)	NS ε	263.0 (140.5-421.3)	256.0 (185-542)	160 (131-420)	NS γ
PRC (uIU/ml)	32.4 (17.7-91.2)	62.4 (17.0-213.9)	NS ε	**59.3 (19.1-226.9)**	**81.6 (28.2-371.8)**	**9.7 (1.0-61.6)**	**0.01 γ**
ARR	7.0 (1.9-11.0)	3.9 (1.2-17.9)	NS ε	3.4 (1.2-13.2)	3.6 (1.0-17.1)	21.1 (4.3-190.0)	NS γ

Table 4-2: Plasma and serum measurements by group.
Data presented as mean (SD) or median (IQR). Comparisons made using t-test (∞) or one-way ANOVA (Δ) for normally distributed data and Mann Whitney U-test (ε) or Kruskall-Wallis test (γ) for non-parametric analysis. Categorical variables were compared using the Chi Square test (δ). P<0.01 = significant (highlighted in bold).

Variable	EH controls N=30	CKD patients N=70	P	DMN N=34	IgAN N=27	MGN N=9	p
U Sod (mmol/l)	80.0 (45.1)	79.3 (33.2)	NS ∞	75.4 (24.2)	79.9 (36.2)	92.1 (51.0)	NS Δ
U Sod (mmol/24h)	155.7 (79.7)	162.9 (70.4)	NS ∞	169.8 (69.4)	149.9 (59.7)	173.5 (100.6)	NS Δ
U Pot (mmol/l)	**41.6 (19.1)**	**29.6 (12.3)**	**0.003 ∞**	27.6 (8.3)	34.0 (15.6)	24.1 (10.5)	NS Δ
U Pot (mmol/24h)	**86.4 (31.1)**	**61.0 (24.1)**	**<0.001 ∞**	60.7 (20.2)	64.0 (27.6)	53.3 (27.5)	NS Δ
QP (g/l)	**0.0 (0.0-0.0)**	**0.5 (0.2-1.1)**	**<0.001 ε**	0.7 (0.2-1.7)	0.3 (0.2-0.7)	0.5 (0.4-1.2)	NS γ
QP (g/24h)	**0.1 (0.1-0.1)**	**1.0 (0.3-2.8)**	**<0.001 ε**	1.5 (0.4-3.6)	0.6 (0.3-1.4)	1.0 (0.4-3.2)	NS γ
uPCR (mg/mmol)	**0.0 (0.0-2.0)**	**75 (21-220)**	**<0.001 ε**	132 (19-270)	48 (19-153)	75 (35-285)	NS γ

Table 4-3: Urine measurements by group.
Data presented as mean (SD) or median (IQR). Comparisons made using t-test (∞) or one-way ANOVA (Δ) for normally distributed data and Mann Whitney U-test (ε) or Kruskall-Wallis test (γ) for non-parametric analysis.

4.3.2 Urine collection adequacy

In this study, 84% of measured creatinine clearance rates were within 35% of the expected creatinine clearance rates. In the majority of patients out with this range (12 patients), the 24h measured creatinine clearance was greater than the expected creatinine clearance, suggesting error relating to the estimation calculation (Cockcroft and Gault) rather than incomplete collections (Figure 4-1) and therefore the collections are valid.

Measured CrCl = ((UCr (mmol/l)*1000) * UVol (ml)) / (Scr (mmol/l) * 1440)

Estimated CrCl (C&G) = ((140-age)*weight (Kg) *1.23 male, 1.04 female) / sCr

A previous study found that in 94% of normal individuals, the measured creatinine clearance will be within ± 35% of the estimated creatinine clearance(261).

4.3.3 Measured levels of urinary steroids, plasma aldosterone and plasma renin concentration in study cohort.

Excretion rates of THALDO, THDOC and cortisol metabolites did not differ between patients with essential hypertension and patients with CKD (Table 4-4). Similarly, there were no significant differences in excretion rates between primary renal diseases (DMN, IGAN or MGN) (Figure 4-1).

PAC and PRC did not differ significantly between patient groups. PRC was significantly lower in patients with membranous nephropathy compared with DMN or IgAN (p=0.01). Three of these patients were prescribed cyclosporine, which ordinarily would increase PRC, but in fact these patients had a PRC of less than 10uIU/ml. If these patients are excluded the median PRC in the membranous group remained significantly lower at 17.5 (7.5-112.9) uIU/ml (p=0.02).

THE levels were higher in essential hypertensives. The ratio of (THF + aTHF) : THE (reflecting hepatic 11βHSD1 activity – see section 1.4.8) was higher in CKD patients. Table 4-5 shows that urinary free cortisone and cortisol measurements, which were available for 78 patients, did not differ significantly between groups, nor did the urinary F:E ratio (predominantly reflecting 11βHSD2 activity).

THS, the derivative of deoxycortisol, and total cortisol metabolite excretion rates did not differ between patients with hypertension and patients with CKD, nor did the ratio of S (deoxycortisol) to F (cortisol).

Variable	Normal range	EH controls N=30	CKD patients N=70	P	DMN N=34	IgAN N=27	MGN N=9	p
THALDO (mcg/24h)	10-120	62.3 (24.0)	56.7 (22.8)	NS ∞	59.1 (25.5)	53.5 (21.6)	56.2 (14.5)	NS Δ
THDOC (mcg/24h)	2-28	75.2 (32.5)	66.1 (23.0)	NS ∞	68.9 (22.5)	62.3 (24.5)	65.8 (21.3)	NS Δ
THS (mcg/24h)	3-130	50.1 (20.7)	43.3 (20.8)	NS ∞	45.7 (21.8)	38.6 (19.2)	46.5 (21.3)	NS Δ
CORT METAB (mcg/24h)		100049 (4985)	8507 (5050)	NS ∞	7425 (3831)	10506 (6442)	7268 (3444)	NS Δ
Ratio (THF + aTHF: THE)		**1.7 (1.5-2.1)**	**2.3 (1.8-4.0)**	**0.001** ε	2.5 (1.8-4.2)	2.1 (1.7-3.9)	2.0 (1.5-3.2)	NS γ
Ratio (THS : THF + aTHF)		0.025 (0.025)	0.021 (0.011)	NS ∞	0.022 (0.007)	0.018 (0.01)	0.029 (0.02)	NS Δ
THE (mcg/24h)	1100-5100	**3297 (2175-4644)**	**2030 (1293-2905)**	**<0.001** ε	1648 (1229-2515)	2388 (1304-3890)	2103 (1385-3271)	NS γ
THF (mcg/24h)	310-3680	2969 (1758-3932)	2235 (1442-3131)	NS ε	2187 (1336-2869)	2574 (1702-3658)	1740 (1169-3066)	NS γ
ATHF (mcg/24h)	490-3480	3112 (1628-4518)	3074 (1671-4694)	NS ε	2541 (1257-4396)	4035 (1970-7062)	3366 (1929-3739)	NS γ
PAC (pmol/l)	100-400 (supine)	236.5 (163.2-331.3)	254.5 (162.3-442.5)	NS ε	263.0 (140.5-421.3)	256.0 (185-542)	160 (131-420)	NS γ
PRC (uIU/ml)	0-40 (supine)	32.4 (17.7-91.2)	62.4 (17.0-213.9)	NS ε	**59.3 (19.1-226.9)**	**81.6 (28.2-371.8)**	**9.7 (1.0-61.6)**	**0.01** γ
ARR	<35	7.0 (1.9-11.0)	3.9 (1.2-17.9)	NS ε	3.4 (1.2-13.2)	3.6 (1.0-17.1)	21.1 (4.3-190.0)	0.05 γ

Table 4-4: Urinary steroid metabolites and plasma RAAS measurements in the whole cohort and stratified by group.
Data presented as mean (SD) or median (IQR). Comparisons made using t-test (∞) or one-way ANOVA (Δ) for normally distributed data and Mann Whitney U-test (ε) or Kruskall-Wallis test (γ) for non-parametric analysis. Categorical variables were compared using the Chi Square test (δ). P<0.01 = significant. Reference range for PRC(278).

Variable	Whole cohort N=78	EH controls N=25	CKD patients N=53	p	DMN N=27	IgAN N=17	MGN N=9	P
F	8.2 (4.4-12.6)	7.0 (4.0-15.7)	8.8 (4.4-12)	NS ε	7.6 (4.2-13.3)	8.8 (6.6-11.0)	10.3 (3.7-12.3)	NS γ
E	31.6 (13.8)	34.6 (13.9)	30.3 (13.6)	NS ∞	34.4 (15.0)	28.3 (9.1)	21.6 (12.7)	NS Δ
F:E	0.3 (0.2-0.4)	0.2 (0.1-0.4)	0.3 (0.2-0.5)	NS ε	0.3 (0.2-0.4)	0.3 (0.2-0.6)	0.5 (0.2-0.9)	NS γ

Table 4-5: Urinary free steroid measurements (mcg/24h) by group.
Data presented as mean (SD) or median (IQR). Comparisons made using t-test (∞) or one-way ANOVA (Δ) for normally distributed data and Mann Whitney U-test (ε) or Kruskall-Wallis test (γ) for non-parametric analysis. Categorical variables were compared using the Chi Square test (δ). P<0.01 = significant.

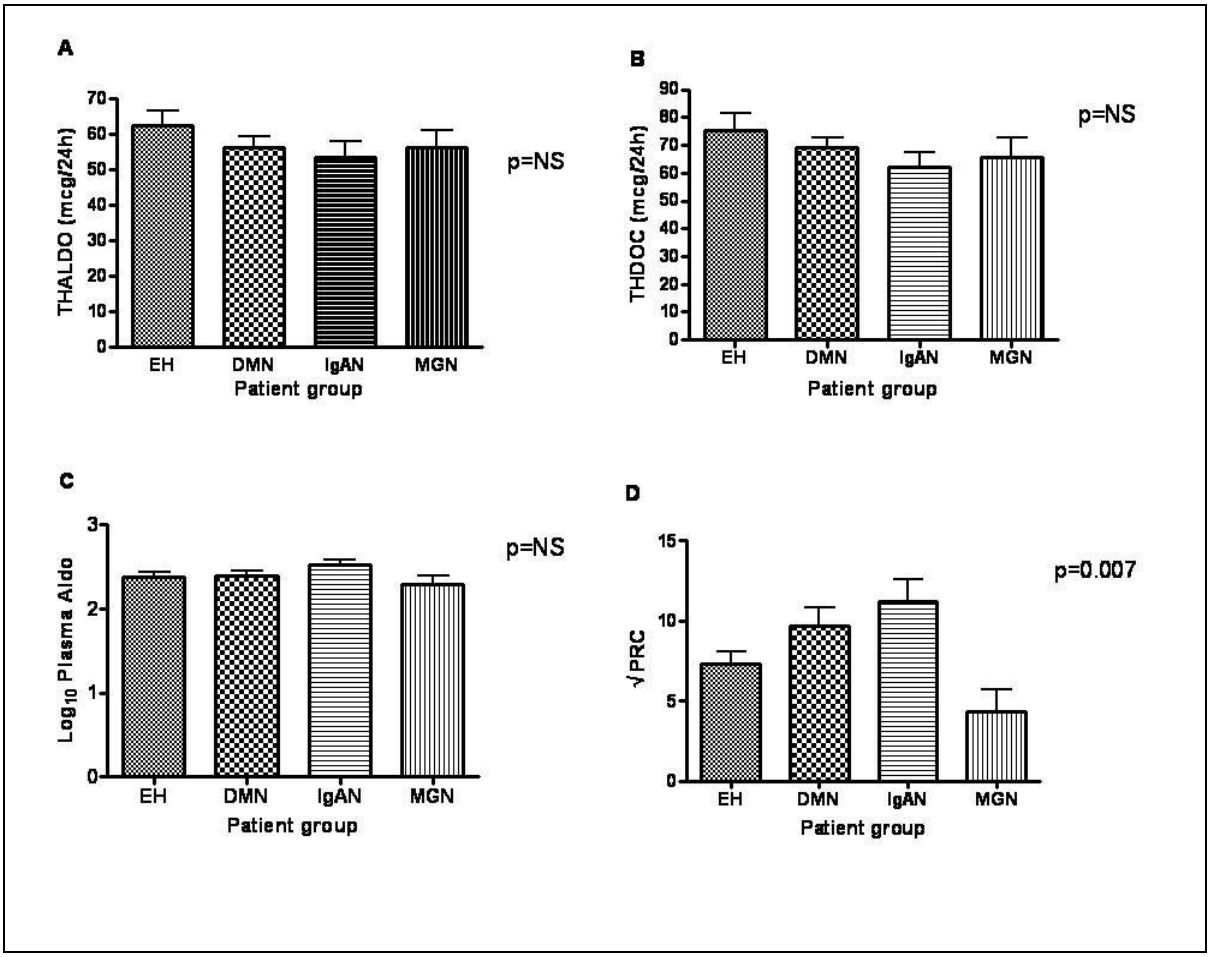


Figure 4-1: Bar chart of mean (SD) levels of steroid and renin by disease group.

THAldo (A), THDOC (B), log PAC (C) and square root PRC (D) concentrations according to patient group. Comparisons made using one-way ANOVA.

4.3.4 Urinary cortisol and cortisone measurements

Seventy-eight patients had measurements of 24h urinary free cortisol (F) excretion rate, of whom 25 had EH and 53 had CKD. Seventy-five had 24h urinary free cortisone (E) excretion rates, of whom 24 had EH and 51 had CKD (Table 4-5). There were no significant differences in measurements of F, E or F:E ratio between patients with CKD or EH. There was no correlation between measurements of F, E or F:E and eGFR, LVMI, 24h UProt, 24h USod, THDOC or THALDO excretion. There was therefore no evidence to support the hypothesis that there is reduced 11BHSD2 activity in CKD.

4.3.5 Association between renal function measurements and THALDO, THDOC, plasma aldosterone and plasma renin concentration.

No significant association was seen between level of renal function, estimated using the MDRD6 formula, and steroid measurements (THALDO, THDOC), PRC or PAC levels, in the cohort as a whole (Figure 4-2). There was a trend towards higher PRC levels with poorer renal function but this did not reach statistical significance ($p=0.053$). When patients prescribed β-blockers were excluded, this relationship was significant ($r=-0.289$, $p=0.02$).

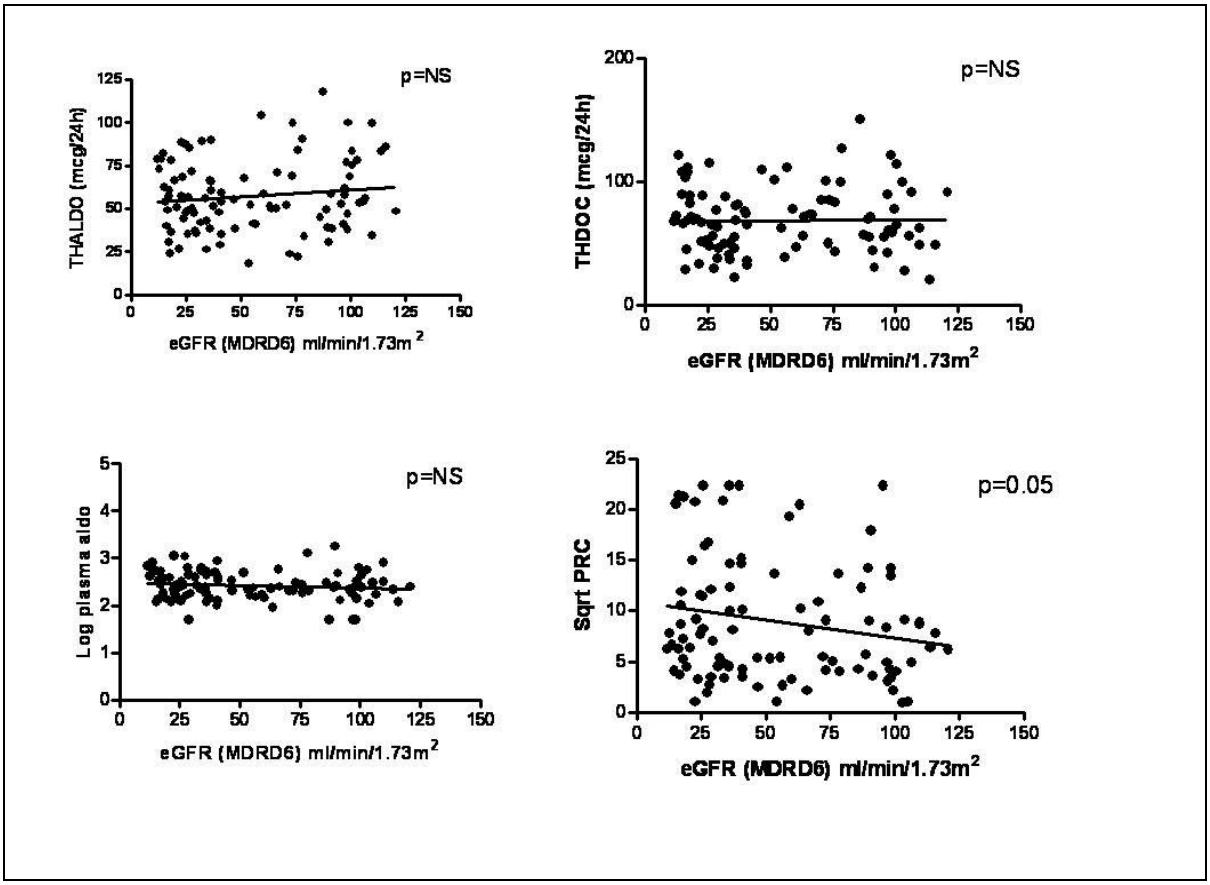


Figure 4-2: Scatterplot of THAldo excretion (A), THDOC excretion (B), PAC (C) and PRC (D) and eGFR with fitted linear regression line and estimate of significance.

4.3.6 Association between prescribed medications and steroid measurements

Drug therapies are described in Table 4-6. CKD patients were prescribed a higher number of anti-hypertensives compared with the hypertensive cohort (2.6 vs. 2.1 p=0.045), and were significantly more likely to be prescribed a loop diuretic, an HMG CoA reductase inhibitor, a β-blocker and an ACE inhibitor. Hypertensive patients were more likely to be prescribed a thiazide diuretic. Within the CKD cohort, patients with IgA nephropathy were less likely to be prescribed an alpha blocker.

4.3.6.1 Urinary steroid excretion and drug prescription

To assess whether different drug classes affected urinary steroid metabolite excretion or plasma measures, levels were compared in patients prescribed these medications versus those not (Table 4-7).

ARB treatment of patients with CKD resulted in significantly higher excretion of THALDO (61.6 vs. 50.4, p=0.018) and THDOC (72.7 vs. 50.4 p=0.042), which was not seen in the EH group. No effect was seen with ACEi in either group. Patients with EH demonstrated a marked increase in THALDO (78.9 vs. 47.3 mcg/24h, p<0.001) and THDOC (97.4 vs. 54.5 mcg/24h, p<0.001) excretion with aspirin treatment but this was not seen in patients with CKD.

4.3.6.2 PAC and drug prescription

PAC was significantly lower in CKD patients prescribed an ACEi (p=0.01; median 217 vs. 415 pmol/l). Prescription of an ACEi did not significantly affect PAC levels in EH patients.

4.3.6.3 PRC and drug prescription

Patients with EH prescribed thiazide diuretics had a higher median PRC (83.8 vs. 24.1uIU/ml, p=0.018), which was not seen in CKD patients, perhaps due to the low thiazide usage in this group. Patients with EH prescribed a statin had a significantly lower median PRC (20.9 vs. 78.7 uIU/ml, p=0.004), reflected in a significantly higher ARR (p=0.002), than patients who were not prescribed a statin.

Clearly, the possibility of interpreting these values is limited because patients were not prescribed individual drugs in isolation and the impact of different drugs on the RAAS varies e.g. loop diuretics increase PRC and plasma Aldo levels whilst ACEi reduce plasma Aldo and increase PRC (see intro section 1.4.10).

4.3.6.4 Independent drug effects on steroid measurements

Looking at all patients together, a multivariate linear regression model was constructed entering all the hypertensive drug classes together for the predicted variable of √PRC. β-blocker treatment resulted in a significantly lower level of PRC (B= -0.21, p=0.04). Undertaking the same analysis for log PAC, ACEi (B= -0.204, p=0.002) and loop diuretics (B= 0.169, p=0.016) were significant independent predictors. No drug significantly predicted THALDO excretion using the same analysis.

DRUG % prescribed	EH N=30	CKD N=70	P	DMN N=34	IGAN N=27	MGN N=9	P
ACE	**36.7**	**64.3**	**0.01**	67.6	63.0	55.6	NS
ARB	43.3	42.9	NS	35.3	48.1	55.6	NS
ACE & ARB	**0**	**25.7**	**0.001**	29.4	18.5	33.3	NS
β blocker	**13.3**	**32.9**	**0.035**	41.2	33.3	0.0	NS
CCB	56.7	44.3	NS	52.9	44.4	11.1	NS
Central	0.0	4.3	NS	2.9	3.7	11.1	NS
α blocker	12	17.1	NS	**26.5**	**0.0**	**33.3**	**0.009**
Loop	**4**	**50**	**<0.001**	55.9	40.7	55.6	NS
Thiazide	**56**	**5.7**	**<0.001**	8.8	3.7	0.0	NS
Aspirin	48	47	NS	55.9	37.0	44.4	NS
Statin	**43**	**68.6**	**0.016**	79.4	63.0	44.4	NS
Total number anti-BP meds	**2.1**	**2.6**	**0.045**	2.9	2.4	2.2	NS

Table 4-6: Drug prescriptions by group – expressed as percentage of patients prescribed. ***Comparisons made using Chi Square test.***

Patient group	*Drug*	*THALDO*	*THDOC*	*PAC*	*PRC*	*ARR*
CKD patients	**ACEi**			0.01		0.03
	ARB	0.02	0.04			
EH patients	**Thiazide**				0.02	
	Aspirin	<0.001	<0.001			
	Statin				0.004	0.002

Table 4-7: Significant differences, expressed as a p value, in measures of urinary steroid metabolites and plasma measures in patients prescribed certain drugs versus those not, stratified by patient group. ***Comparisons made by t-test or Mann Whitney U test as appropriate.***

4.3.7 Correlations between steroid and RAAS measurements and clinical measurements

Levels of THALDO and THDOC excretion, cortisol metabolites, PAldo, PRC and ARR were entered into a correlation matrix with the following demographic and laboratory markers: age, sex, weight, waist circumference, SBP, DBP, AIx, AIx@75bpm, ECG abnormalities, PWV, RHI, EF, EDV/BSA, ESV/BSA, LVMI, eGFR (MDRD6), CRP, urate, total cholesterol, TG, HDL, LDL, C/HDL ratio, AdCal, phosphate, PTH, vitamin D, Hb, 24h QP, PCR, USod (mmol/24h) and UPot (mmol/24h). Spearman's or Pearson's correlation coefficients are reported as appropriate with $p<0.01$ deemed as significant. There were significant differences between groups in terms of significant correlates (Tables 4-8 and 4-9).

THALDO and THDOC excretion rates correlated significantly positively with 24h USod excretion and 24h UPot excretion in CKD patients, but not in EH. There was also a strong correlation between THALDO and THDOC excretion and urinary protein excretion and LVMI in patients with CKD. In EH patients the strongest correlate with THALDO and THDOC excretion was serum parathyroid hormone.

Plasma aldosterone concentration correlated significantly with LVMI and female gender in EH but not CKD.

In EH patients PRC correlated significantly with thiazide diuretic and statin usage. In CKD, PRC was inversely correlated with SBP, DBP, serum sodium and AIx@75bpm. ARR correlated more closely with PRC than PAC in both groups, suggesting PRC is a stronger determinant. Correlations with the ARR reflected those seen with PRC.

Excretion of cortisol metabolites correlated significantly with weight in both groups and waist circumference in EH. There was also a positive correlation with 24h USod in CKD patients and cortisol metabolite excretion correlated with EDV/BSA and AIx@75bpm in CKD patients.

EH patients N=30	THAldo	THDOC	Cort Metab	PAC	PRC	ARR
THAldo	1	0.963 <0.001				
THDOC	0.963 <0.001	1				
PAC						0.517 0.004
PRC						-0.839 <0.001
Age						
Male sex				-0.537 0.002		
Weight			0.623 <0.001			
Waist circumf.			0.572 0.001			
SBP						
DBP						
Serum Na						
PTH	-0.673 <0.001	-0.625 0.001				
C/HDL						
UNa (mmol/24h)						
UPot (mmol/24h)						
uPCR						
24h QP						
EDV/BSA						
LVMI				0.57 0.001		
AIx@75bpm						
Thiazide diuretic				0.523 0.009		
Statin therapy				-0.512 0.005		

Table 4-8: Correlation matrix including EH patients.
Only significant correlations reported. Data expressed as r (correlation coefficient) and p (significance).

CKD patients N=70	***THAldo***	***THDOC***	***Cort Metab***	***PAC***	***PRC***	***ARR***
THAldo	1	0.917 <0.001				
THDOC	0.917 <0.001	1				
PAC						0.424 <0.001
PRC						-0.907 <0.001
Age						
Male sex						
Weight			0.404 0.001			
Waist circumf.						
SBP					-0.485 <0.001	0.395 0.001
DBP					-0.31 0.009	
Serum Na					-0.338 0.004	0.326 0.006
PTH						
C/HDL						
UNa (mmol/24h)	0.614 <0.001	0.526 <0.001	0.331 0.007			
UPot (mmol/24h)	0.538 <0.001	0.481 <0.001				
uPCR	0.354 0.004	0.32 0.009				
24h QP	0.426 <0.001	0.371 0.002				
EDV/BSA			0.383 0.004			
LVMI	0.43 0.001	0.447 0.001				
AIx@75bpm			-0.388 0.002		-0.359 0.001	
Thiazide diuretic						
Statin therapy						

Table 4-9: Correlation matrix including CKD patients.
Only significant correlations reported. Data expressed as r (correlation coefficient) and p (significance).

4.3.8 Relationship between urinary steroid excretion and urinary electrolytes

4.3.8.1 Urinary sodium excretion and steroid excretion rates

Aldosterone promotes sodium reabsorption and a high plasma sodium should suppress aldosterone release. In patients with CKD, THALDO and THDOC each strongly positively associated with urinary sodium excretion (THALDO R^2 = 0.38, THDOC R^2=0.28) (Figure 4-3). In patients with EH no relationship was seen between THALDO excretion and urinary sodium.

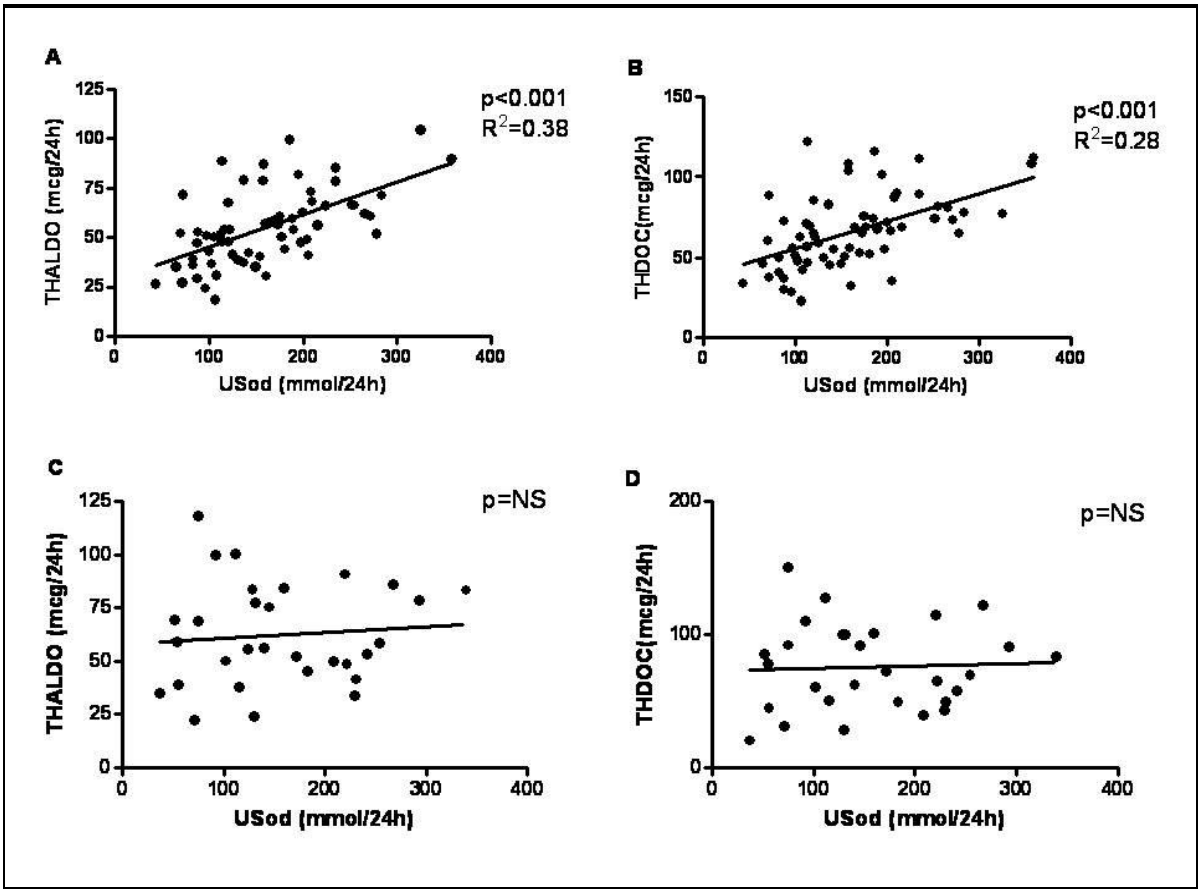


Figure 4-3*:* Scatterplot of urinary sodium excretion (mmol/24h) versus THAldo or THDOC excretion, with fitted linear regression line and estimate of significance.
A and B = CKD patients, C and D = EH patients.

4.3.8.2 Urinary potassium and urinary steroid excretion rates

In both groups, a higher 24h UPot excretion was associated with a higher 24h THAldo excretion rate (CKD patients R^2=0.36, p<0.001 and EH patients R^2=0.15, p=0.04) (Figure 4-4).

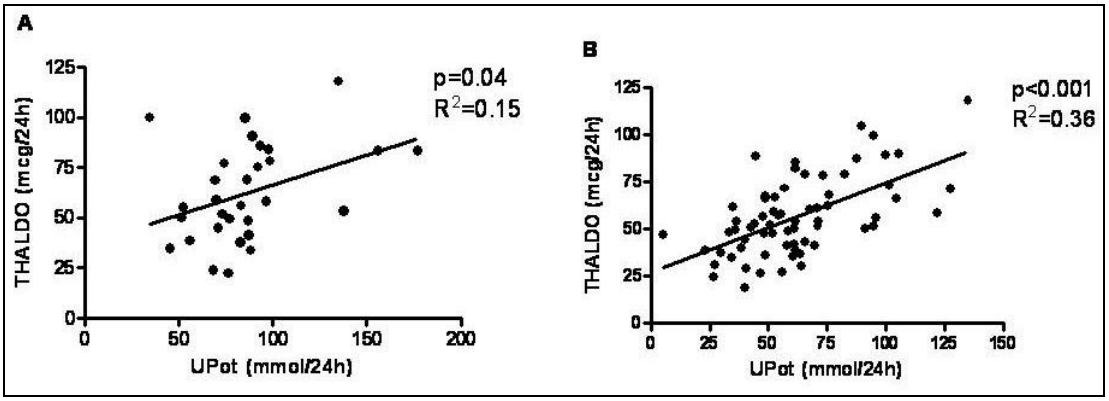


Figure 4-4*:* Scatterplot of THAldo excretion versus urinary potassium excretion (mmol/24h) with fitted linear regression line and an estimate of significance. A = EH patients; B = CKD patients.

4.3.8.3 THE, THF and THS and urinary electrolyte excretion

If excretion rates of THAldo and THDOC were simply a reflection of solute drag in patients with CKD, then THE, THF and THS would similarly exhibit correlations with urinary electrolytes. In fact, there was no correlation between these measures with urinary potassium and only a weak correlation with urinary chloride (r=0.28 p=0.02) and urinary sodium (r=0.356, p=0.03) in patients with CKD. In patients with EH, no correlation was seen between urinary electrolytes and urinary cortisol metabolites.

4.3.9 The relationships between steroid levels and traditional trophins.

To determine whether PRC, serum potassium, serum sodium and ACTH are associated with Aldo levels, linear regression models were constructed for patients with EH and patients with CKD.

4.3.9.1 PRC

No significant relationship was seen in CKD patients between PRC and PAC, THALDO or THDOC excretion (Figure 4-5). There was a non-significant trend towards a higher level in the three variables with higher PRC in patients with EH (p=0.2).

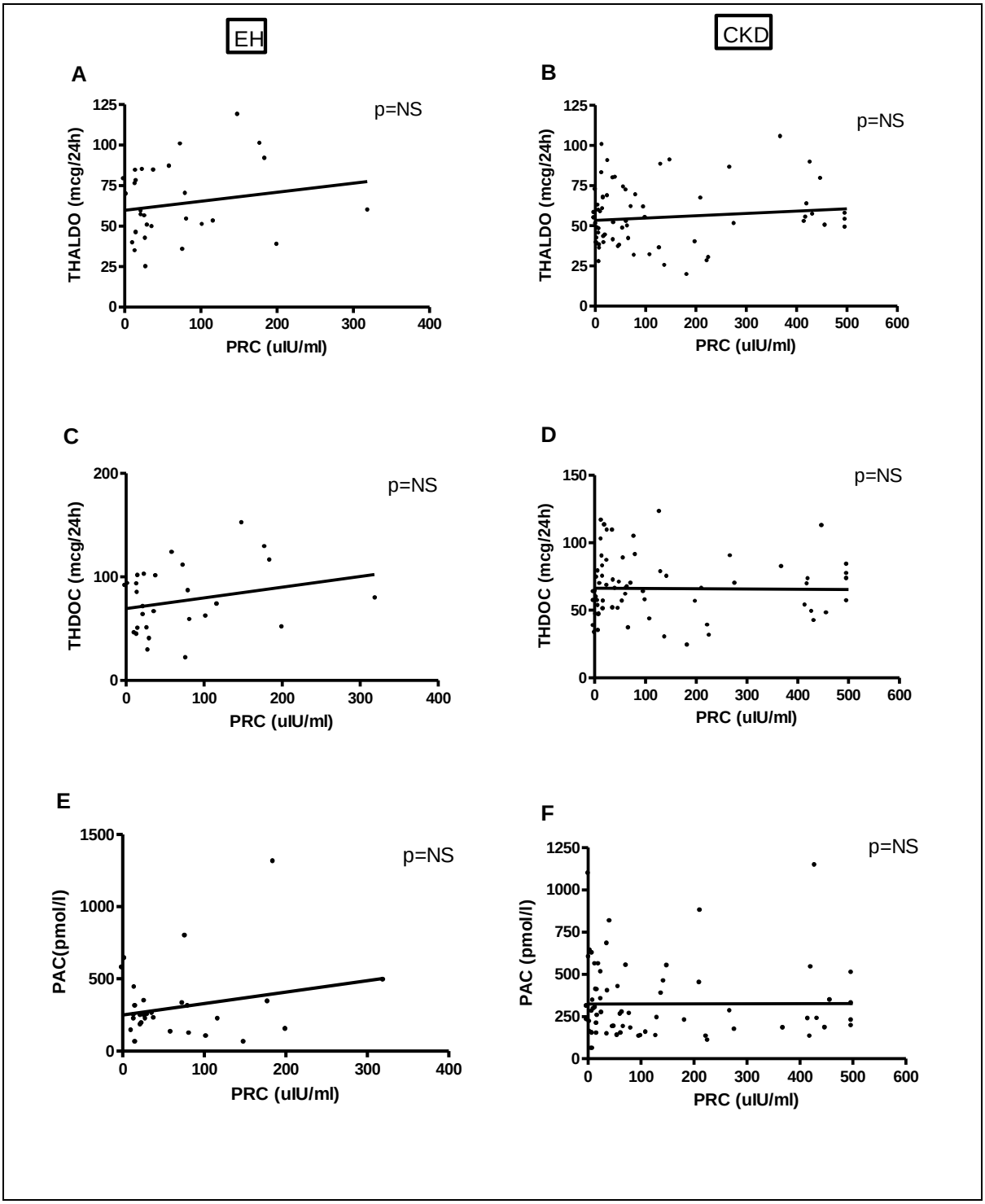


Figure 4-5: Scatterplot of PRC and THAldo, THDOC and PAC.

A,C and E = EH patients. B,D and F = CKD patients. Fitted linear regression line and estimates of significance.

4.3.9.2 Serum potassium

In patients with CKD, a higher serum potassium was associated with a lower PAC, contrary to what would be expected. When patients prescribed an ACEi were excluded, no relationship was seen. In patients with EH there was no relationship (Figure 4-6). Serum potassium was not associated with THALDO excretion in either group.

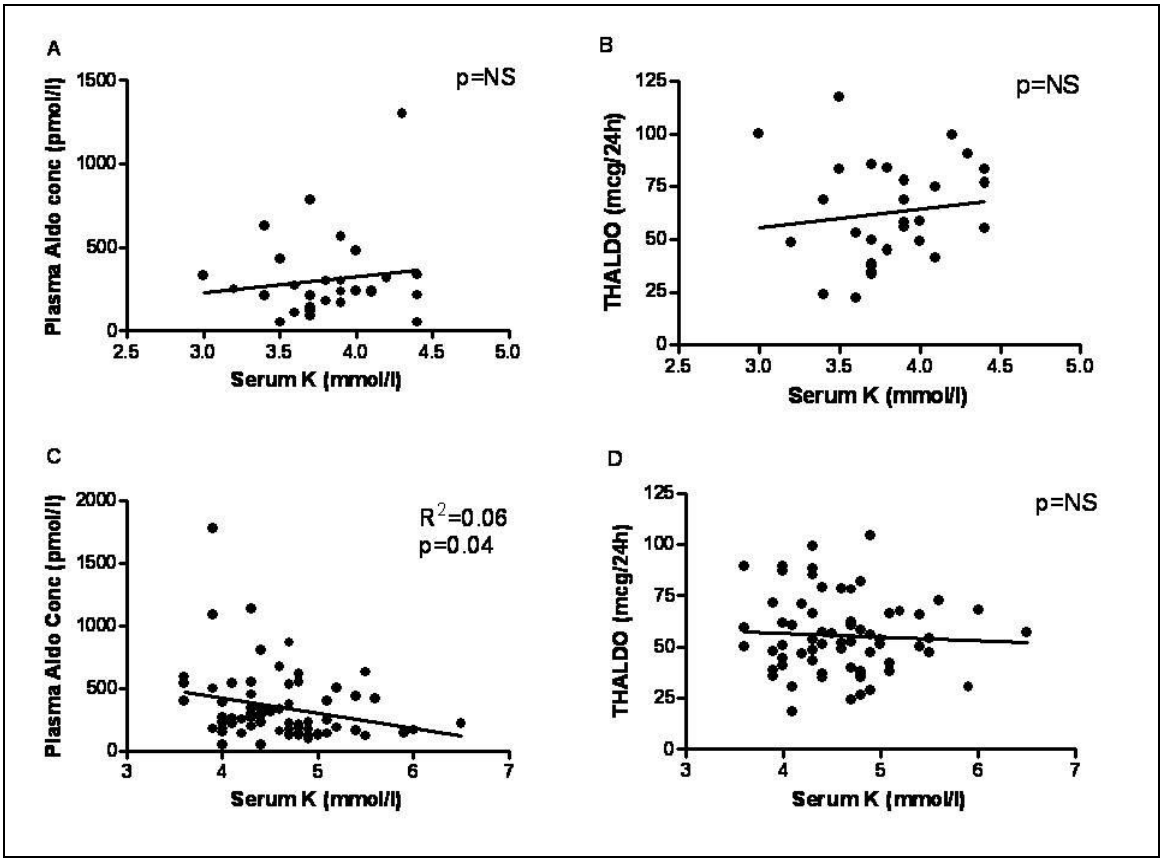


Figure 4-6: Scatterplot of serum potassium versus PAC and THAldo excretion with fitted linear regression line and estimate of significance.

A & B = EH .C & D = CKD.

4.3.9.3 ACTH

In EH patients, there was a trend towards a negative correlation between THAldo excretion and cortisol metabolite excretion (a surrogate for ACTH stimulation) (p=0.06). In patients with CKD the contrary was seen, with a positive correlation between cortisol metabolites and THAldo (p=0.05). (Figure 4-7).

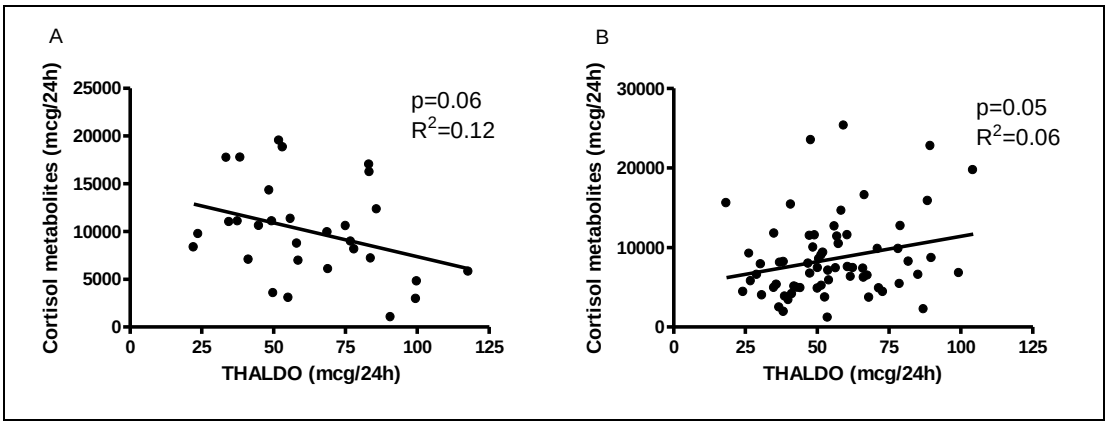


Figure 4-7: Scatterplot of THAldo and cortisol metabolite excretion with fitted linear regression line. ***A=EH patients. B=CKD.***

4.3.9.4 Relationship between PRC and plasma sodium concentration

In patients with EH and CKD, PRC was significantly lower in patients with a high plasma sodium (Figure 4-8).

4.3.9.5 Relationship between PAC and plasma sodium concentration

There was no relationship between PAC and plasma sodium concentration in patients with EH or patients with CKD.

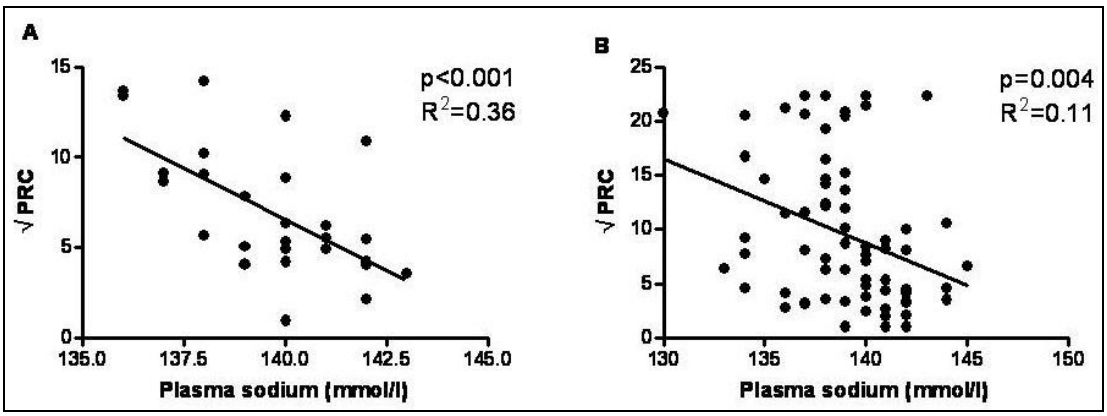


Figure 4-8: Scatterplot of plasma sodium concentration and √ plasma renin concentration, with fitted linear regression line and estimate of significance.
A = EH patients, B = CKD patients.

4.3.10 Determinants of THALDO and THDOC excretion in patients with CKD

Significant correlates with THALDO and THDOC were entered separately into a linear regression model to assess determinants of excretion. Due to high collinearity between THALDO and THDOC they were not entered together into subsequent multivariate models. The measure of proteinuria which correlated most closely with THALDO and THDOC excretion (24h QP) was entered into the model.

Significant univariate predictors of THALDO excretion were THDOC excretion, 24h urinary sodium and potassium, 24h QP and LVMI. Significant independent predictors of THALDO excretion on multivariate analysis were 24h urinary sodium and potassium excretion (Table 4-10).

Similarly, significant univariate predictors of THDOC excretion were THALDO excretion, 24h urinary sodium and potassium, 24h QP and LVMI. Significant independent predictors of THDOC excretion on multivariate analysis were 24h urinary sodium and potassium excretion and logLVMI (Table 4-11).

Variable	UNI B	95% CI	β	P	MULTI B	95% CI	β	p
USod (mmol/24h)	0.17	0.11-0.22	0.61	<0.001	0.10	0.03-0.17	0.38	0.004
U Pot (mmol/24h)	0.43	0.26-0.60	0.54	<0.001	0.24	0.04-0.43	0.30	0.02
THDOC	0.77	0.68-0.85	0.92	<0.001	-	-	-	-
Log 24h QP	13.03	5.3-20.7	0.39	0.001	-	-	-	-
Log LVMI	66.10	17.3-115.0	0.35	0.009	-	-	-	-

Table 4-10: Univariate (UNI) and multivariate (MULTI) linear regression model for predicted variable THAldo excretion.
Significant correlates entered as univariate predictors. R-square for multivariate model 0.492, p<0.001

Variable	UNI B	95% CI	β	P	MULTI B	95% CI	β	p
THALDO	1.10	0.98-1.22	0.92	<0.001	-	-	-	-
USod (mmol/24h)	0.17	0.10-0.24	0.53	<0.001	0.091	0.003-0.18	0.28	0.04
U Pot (mmol/24h)	0.46	0.25-0.67	0.48	<0.001	0.285	0.04-0.54	0.30	0.03
Log LVMI	90.8	33.57-148.07	0.40	0.002	63.2	4.34-122.1	0.28	0.04
Log 24h QP	13.92	4.52-23.31	0.35	0.004	-	-	-	-

Table 4-11: Univariate (UNI) and multivariate (MULTI) linear regression model for predicted variable THDOC excretion.
Significant correlates entered as univariate predictors. R-square for multivariate model 0.417, p<0.001.

4.3.11 Aldosterone synthase C-344T genotype frequency and association with demographics

63 patients had suitable blood samples for genotyping and were genotyped for the C-344T aldosterone synthase polymorphism. It can be seen that there was no difference in genotype frequency between the patients with CKD and the patients with EH (Table 4-12).

There was no significant difference in PAC or THALDO excretion rates between genotype groups. Similarly there was no difference in LVMI, QP, cAIx, RHI, PWV or urinary sodium between genotypes.

Genotype	**All** **N=63**	**CKD** **N=40**	**EH** **N=23**
CT	33 (52.4%)	21 (52.5%)	12 (52.2%)
CC	14 (22.2%)	9 (22.5%)	5 (21.7%)
TT	16 (25.4%)	10 (25%)	6 (26.1%)

Table 4-12: Genotype frequency for the C-344T polymorphism by patient group.

4.3.12 Predictors of proteinuria in patients with CKD

Significant correlates (Spearman's (r,p)) with 24h QP in patients with CKD were: weight (0.254, p=0.04), SBP (0.407, p=0.001), pulse pressure (0.318, p=0.008), eGFR (-0.271, p=0.03), phosphate (0.289, p=0.02), 24h USod (0.317, p=0.009), THDOC (0.371, p=0.002) and THAldo (0.426, p<0.001) (Figure 4-9).

The significant correlates were entered separately into univariate linear regression models for prediction of log 24hQP and all were significantly predictive (Table 4-13). Two multivariate models were then constructed, entering THAldo and THDOC excretion separately with the other significant predictors. This showed that THAldo and SBP were the only significant independent predictors of proteinuria excretion. THDOC was not a significant independent predictor (Table 4-13).

In patients with hypertension, proteinuria levels were very low and in the absence of microalbuminuria measurements, difficult to interpret. The only significant independent predictor of proteinuria in these patients was eGFR.

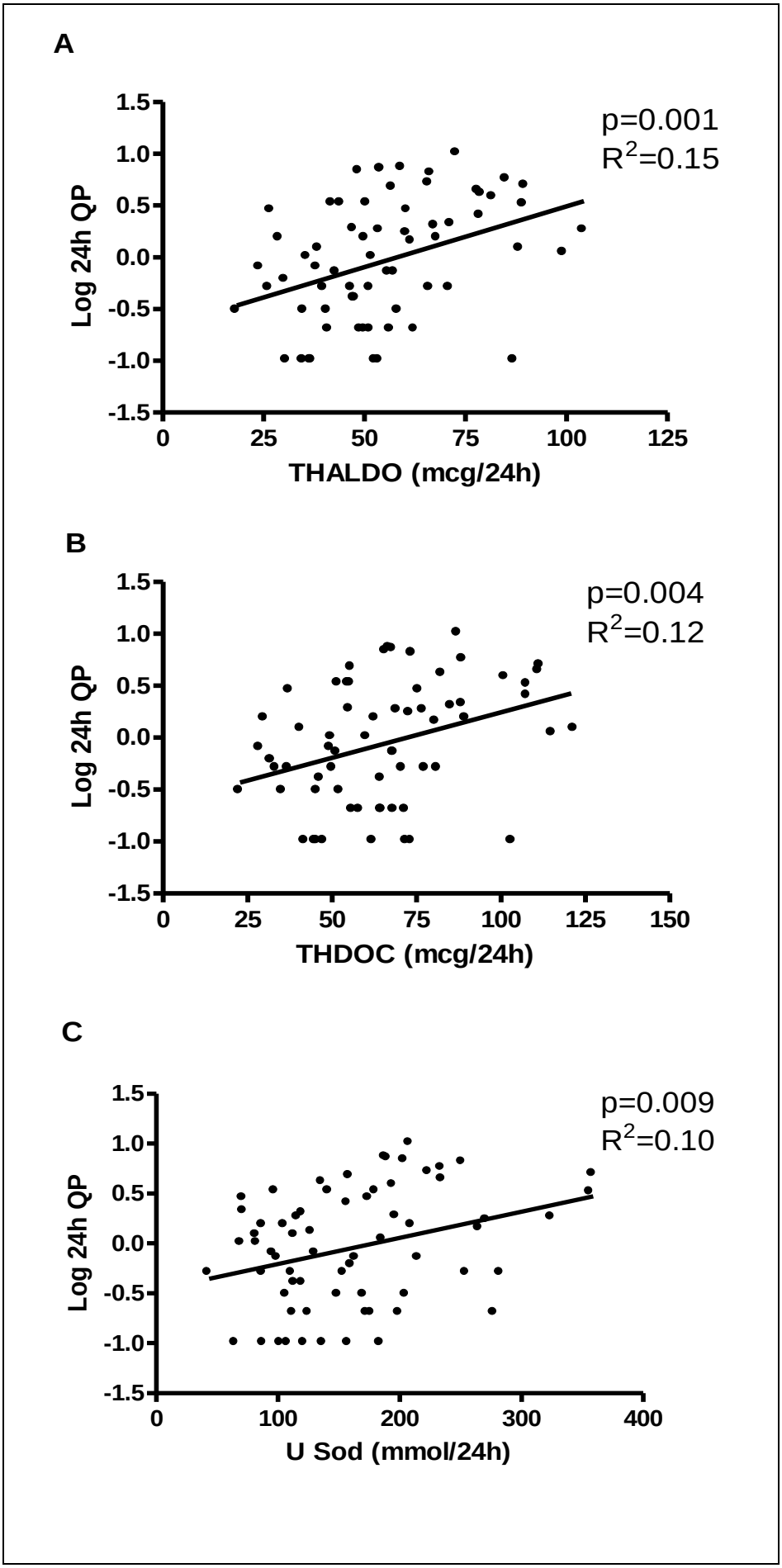


Figure 4-9: Scatterplot of THAldo (A), THDOC (B) and U Sod (C) versus log of 24h quantified proteinuria with fitted linear regression line and estimate of significance.

Variable	UNI B	95% CI	β	P	MULTI B	95% CI	β	p
THALDO	0.01	0.005-0.02	0.39	0.001	0.008	0.0-0.02	0.28	0.04
SBP	0.01	0.003-0.02	0.36	0.003	0.009	0.004-0.015	0.365	0.001
THDOC	0.01	0.003-0.02	0.35	0.004	-	-	-	-
24h USod	0.003	0.001-0.005	0.32	0.009	-	-	-	-
eGFR	-0.006	-0.01 – 0.001	-0.27	0.03	-	-	-	-
Weight	0.01	0.001-0.01	0.26	0.03	-	-	-	-
Phosphate	0.69	0.10-1.29	0.28	0.02	-	-	-	-

Table 4-13: Univariate (UNI) and multivariate (MULTI) linear regression models for prediction of log 24h QP.
Due to multicollinearity, THAldo and THDOC were entered separately. R^2 value for THALDO multivariate model 0.375.

4.3.13 Proteinuria in patients with CKD : Interaction between steroids and sodium

Previous studies have suggested that aldosterone and sodium may interact synergistically to increase proteinuria excretion. Figure 4-10 demonstrates graphically that patients in the highest tertile of THAldo excretion and highest tertile of USod excretion had the highest median 24h QP. A 2-way ANOVA was carried out which revealed that there was no statistically significant interaction between tertiles of USod and THAldo excetion and level of proteinuria.

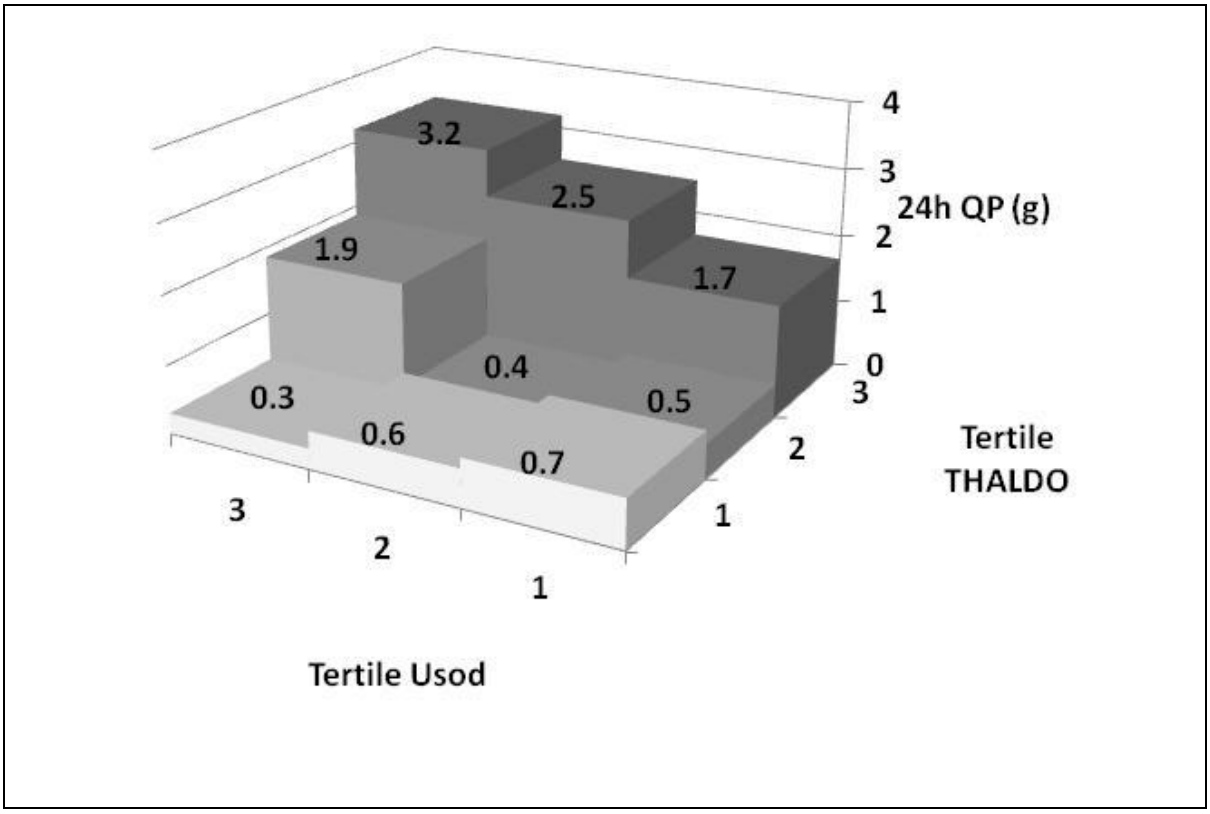


Figure 4-10: 3D bar chart of median 24h QP by tertile of urinary sodium and tertile of THALDO in CKD patients.
Tertile 1 = low, tertile 3 = high.

4.3.14 Relationship between mineralocorticoids, renin and blood pressure

No correlation was seen between urinary mineralocorticoid excretion (THALDO, THDOC), PAC and blood pressure (SBP, DBP) in CKD or EH patients. Similarly, there was no relationship between PRC and blood pressure in EH (Figure 4-11). In CKD, PRC was inversely related to SBP ($r= -0.485$, $p<0.001$) and DBP ($r= -0.31$, $p=0.009$). This relationship was strengthened when patients prescribed β-blockers were excluded.

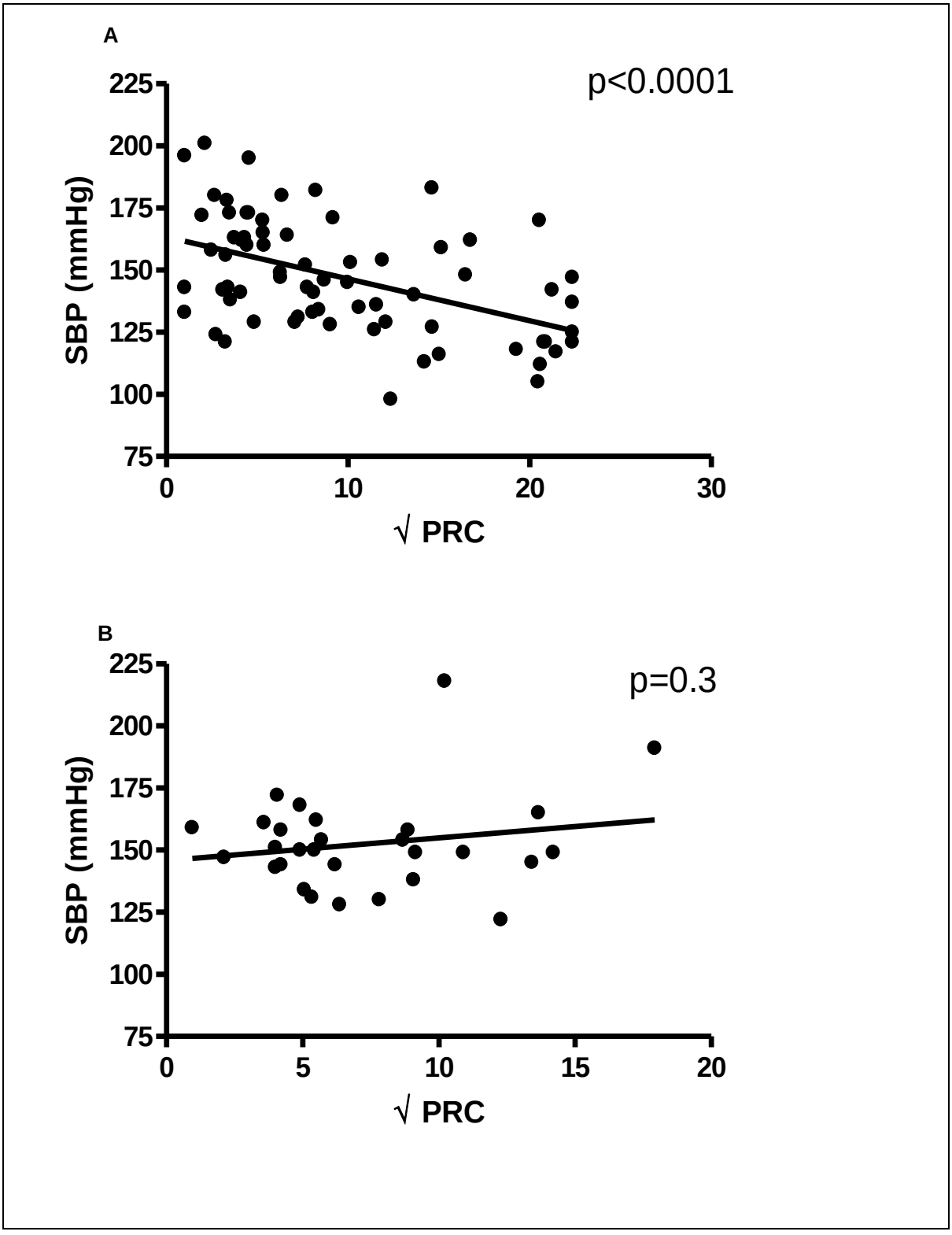


Figure 4-11: Scatterplot of the square root of plasma renin concentration versus systolic blood pressure.

Patients with CKD (A) and EH (B) with fitted linear regression line and estimate of significance.

4.4 Discussion

This study demonstrates a number of novel findings in relation to steroid levels, metabolism and effects in chronic kidney disease. The addition of a well-matched essential hypertensive group allows for the effects of hypertension to be controlled for. There were differences in drug prescriptions between the two groups which should be borne in mind when interpreting the data.

The key findings were:

1. Excretion rates of steroid metabolites and plasma concentrations of aldosterone and renin do not differ between patients with CKD and patients with EH. Levels also do not differ between patients with primary (IgAN and MGN) and secondary (DMN) renal disease.

2. The F:E ratio is not altered in patients with CKD suggesting that 11βHSD2 activity is not reduced.

3. Urinary sodium excretion is a strong independent predictor of THALDO and THDOC excretion in patients with CKD. This relationship is novel and not seen in patients with EH.

4. Urinary potassium excretion is directly proportional to THALDO excretion in patients with CKD and patients with EH.

5. Traditional adrenal cortical trophins do not exhibit predicted effects in CKD. PRC does not influence measures of aldosterone (PAC, THALDO excretion) in patients with CKD but in patients with EH there is a trend towards a directly proportional relationship. Serum potassium concentration is inversely related to PAC in patients with CKD but not EH.

6. Plasma renin concentration was inversely proportional to plasma sodium in patients with CKD and patients with EH.

7. Plasma renin concentration was inversely proportional to systolic and diastolic blood pressure in patients with CKD but not EH. This relationship persisted when patients prescribed beta-blockers were excluded.

8. THALDO excretion, with SBP, is an independent predictor of proteinuria excretion in patients with CKD. An interaction between urinary THALDO excretion, urinary sodium excretion and urinary protein excretion was demonstrated in that patients in the highest tertile of THALDO excretion and highest tertile of urinary sodium excretion had the highest amount of proteinuria excretion.

9. Cortisol metabolite excretion was not associated with urinary electrolyte excretion in patients with CKD or patients with EH. Cortisol metabolites had no association with left ventricular mass or proteinuria excretion in patients with CKD or EH.

4.4.1 Steroid measurement methods and levels in CKD

Previous evidence suggests that 24h excretion of steroid metabolites is more reflective of steroid hormone status than random plasma measurements (101). This study was not designed to test this, but did show that urinary excretion rate of aldosterone and deoxycorticosterone is of more prognostic significance than plasma concentrations, in that THALDO and THDOC excretion rates correlate significantly with pathological features (proteinuria excretion and left ventricular mass index) in patients with CKD whereas plasma aldosterone and plasma renin concentrations did not.

There is no evidence from this study that levels of urinary steroid excretion, plasma aldosterone or plasma renin concentrations relate to eGFR in patients with CKD stages 2-4 in the context of current antihypertensive treatments. This study also demonstrates that there were no differences in steroid measurements between patients with CKD and patients with EH, or patients with diabetic renal disease and patients with primary glomerular disease. This is contrary to what one would expect in CKD where an intravascular overload is presumed, and hypertension and elevated serum potassium which should suppress trophic factors.

Our findings differ from previously published data showing that PAC levels were higher with poorer renal function. Hene et al(120) showed that PAC increased with declining renal function in patients on no medications and with primary renal disease. The increase was most marked when CrCl was between 3-10ml/min. Our study did not include patients with such significantly impaired kidney function as this and this may explain why this association was not found.

A more recent study by Hammer et al(279) is the only comparable study which reports urinary steroid excretion in patients with CKD. There are differences between the Hammer cohort and this study cohort - Hammer assessed 112 patients with non-diabetic, minimally proteinuric renal disease with well preserved renal function (CKD 3). My patients had CKD 3 or 4, a median of 1g proteinuria per day and half had diabetic nephropathy. Hammer et al reported THALDO excretion was not associated with eGFR, SBP or DBP but did find that PAC correlated weakly with THALDO excretion, which was not seen in this cohort. In terms of drug effects, in their cohort ACEi/ARB treatment had no effect on PAC, PRA or THALDO excretion, unlike my cohort where PAC was lower in patients prescribed an ACEi and THALDO excretion was higher in patients prescribed an ARB. They did not assess determinants of steroid excretion or report pathological correlates. The frequency of aldosterone synthase genotype (C-344T) did not differ between patients with CKD and those without and had no impact upon steroid levels in this cohort.

In patients with type 1 diabetes and normal renal function, levels of aldosterone and renin were found to be significantly higher than in normal patients on a high salt diet(280). The failure of this study to demonstrate higher levels of these measures in patients with diabetic nephropathy may reflect the presence of renal disease, the inclusion of patients with type 2 diabetes, or that the control group were not ‘normal’ or alternatively the modulating effects of medications.

4.4.2 Regulation of mineralocorticoid synthesis and the RAAS in CKD

In this study, the regulation of aldosterone synthesis appears to differ from expected physiological principles in patients with CKD and patients with EH. In patients with CKD, the strongest independent determinant of THALDO and THDOC excretion was 24h urinary sodium excretion rate. This is a novel and unanticipated finding and was not seen in patients with EH alone, which is in agreement with other groups’ findings (281).

The observed relationship between urinary electrolyte excretion and urinary steroid excretion in CKD patients could simply reflect an increase in non-selective glomerular permeability. THALDO and THDOC excretion is closely correlated with USod and UPot, and to a lesser extent U Chloride and U Creatinine. However, if the test simply measures molecules (THALDO, THDOC) which are ‘dragged along’ with electrolytes, then one would expect the same picture with THE, THF and THS, which is not seen.

Similarly, the relationship is not seen in patients with EH and therefore it is particular to CKD. Further validation of the use of THALDO / THDOC is provided by their expected close correlation in both cohorts. Furthermore, THS correlates with its metabolites THE and THF. Finally, could the relationship be an artefactual finding based on urine volume? This would not appear to make sense as both measures (urinary steroid and urinary electrolytes) are reported over 24h and derived from the same urine volume, so correlating the two measures will obviate this relationship. Therefore, in chronic kidney disease, excretion of THALDO and THDOC is closely associated with urinary electrolyte excretion, particularly sodium. This relationship is not seen with total cortisol metabolites.

Explanation of these novel findings is potentially multi-fold. It may be that adrenal Aldo production in CKD is more closely regulated by other factors such as catecholamines, endothelin and arginine vasopressin(282) than traditional trophins such as AgII, volume and potassium, which seems unlikely. Alternatively the lower plasma pH in patients with CKD may modulate Aldo production or adrenal response to stimuli. The TASK (TWIK-related acid-sensitive K) channel is found in the zona glomerulosa and activity is significantly reduced at lower pH(283). TASK knockout mice demonstrate zona glomerulosa depolarization and increased Aldo production in response to stimuli(284).

Local Aldo production is also known to occur in the kidney and vasculature but production rates are very small and it is unlikely to be detected by serum or urine analysis. It may be that these systems are responsible for the pathological effects mediated via the MR and are regulated differently to traditional adrenal cortical production.

No other studies report determinants of THDOC excretion in patients with CKD and no studies have analysed the relationship between urinary electrolytes and mineralocorticoids in patients with CKD(281).

Utilising UFF:UFE is a more accurate assessment of the 11βHSD2 enzyme than THF:THE, which mainly reflects 11βHSD1(97). We found no association between level of renal function and F:E ratio, which was similar to findings of a small study of patients with nephrotic syndrome(98). Quinkler et al(99) however found that the F:E ratio (measured using GCMS) increased as eGFR declined in patients with CKD, supporting a loss of 11βHSD2 function. These results are contrary to our findings.

There was no relationship between THALDO excretion and PAC, perhaps reflecting the snapshot view PAC provides, rather than an integrated 24h measure. THALDO did not correlate with PRC (a surrogate for angiotensin II) in patients with EH or CKD, suggesting the adrenal cortex may be less sensitive to AgII in the context of hypertension, renal failure or drug therapy.

PRC was inversely correlated with a higher plasma sodium in both EH and CKD, suggesting that the juxtaglomerular apparatus still senses intravascular volume status as one would expect as renal function declines. This supports older research where salt loading in patients with CKD or patients on HD was associated with suppression of plasma renin activity(285).

Contrary to what might be expected, PAC was inversely related to serum potassium in CKD and there was no significant relationship in EH patients. There was a trend towards an inverse relationship between THALDO excretion and cortisol metabolites (a surrogate for ACTH) in patients with CKD and no significant relationship in patients with EH.

Data with which to compare our findings are sparse. Most existing literature regarding determination of aldosterone levels in CKD relates to patients with more advanced renal disease and describes aldosterone responses to administered stimuli.

Berl and Katz in 1978 (121) showed that eight patients with advanced renal failure responded to marked sodium restriction or the assumption of an upright posture with a rise in PAC, i.e. they responded appropriately to stimuli despite advanced renal failure.

More recently, Bomback(286) assessed haemodialysis patients and healthy volunteers and found that in haemodialysis patients serum potassium did not influence serum aldosterone levels, unlike in my CKD cohort. They also established that, in healthy volunteers, sodium loading led to increased extracellular volume (as assessed by bioimpedence) and lower PAC. In HD patients intradialytic weight gain resulted in increased ECV and suppressed PAC, but to a much lesser degree than in normal controls, i.e. the response was blunted.

Kohagura found that the determinants of PAC in HD patients were serum potassium and renin(122). They also reported that 50% of patients had elevated PAC but this was not associated with a worse mortality risk.

The majority of patients included in this study were overweight, with a mean BMI of 29.3Kg/m^2. Adipocytes are known to release potent mineralocorticoid releasing factors and insulin resistance and hyperinsulinaemia stimulate aldosterone synthesis in vitro. Therefore, obesity may be modulating some of the findings in these patients, although there was no significant difference in BMI between the two groups.

4.4.3 Steroid excretion levels, blood pressure and proteinuria

No relationship was seen between urinary steroids or PAC and blood pressure in either patients with CKD or patients with EH. This may be due to the modulating effects of antihypertensive medications and was also found by Hammer et al(287). In patients with EH, there was no relationship between blood pressure and PRC but in CKD PRC was inversely proportional to SBP and DBP. This finding again suggests that the renin-angiotensin functions differently in CKD compared with patients with EH. Urinary steroid excretion correlated with LVMI, which is the focus of chapter 5.

The degree of proteinuria excretion is an independent predictor of both progression of renal disease and mortality in patients with CKD. THALDO, THDOC and 24h USod excretion were shown to be significant univariate predictors of proteinuria excretion in this cohort, with THALDO excretion and SBP being the only significant multivariate predictors. This has not been demonstrated in patients with CKD before and lends weight to MR blockade as treatment for proteinuria reduction.

There was a suggestion of a relationship between USod, THALDO and proteinuria excretion, although this was not significant on 2-way ANOVA. Using linear regression THALDO / THDOC and 24h urinary sodium are independent predictors of proteinuria, although the steroid is a stronger predictor. It may be that a larger sample size is needed to assess the effect.

4.4.4 Limitations

The study findings are limited by the fact that this was a heterogenous cohort and patients were prescribed multiple different medications, a number of which are known to impact upon aldosterone or renin production. It would however be unethical to perform a study of this nature in CKD in a drug free environment. This may explain why there was no association seen between renal function, renin and aldosterone and why THALDO and THDOC excretion is perhaps higher than one would expect for the level of hypertension

and renal failure. It is difficult to comment on the role of intravascular volume in determining steroid excretion as this is difficult to measure. Similarly, measurement of total body sodium would be of value. Lastly, it would have been useful to include a normal control group receiving no medication.

4.5 Conclusion

This study is the first to compare urinary steroid production in patients with CKD and patients with EH. This novel comparison allows for the effects of hypertension and medication to be controlled for and clearly demonstrates that regulation of steroid production differs in CKD. The usual regulatory mechanisms of serum potassium and angiotensin II appear to be uncoupled in patients with CKD and the strong association between urinary sodium excretion and THALDO and THDOC excretion suggests that alternative regulatory mechanisms are involved. USod is directly proportional to urinary steroid excretion. This is counter-intuitive when the usual physiological role of mineralocorticoids is considered. Conventionally, mineralocorticoids increase expression of the epithelial sodium channel and encourage reabsorption of sodium. Therefore, a negative relationship between urinary sodium levels and urinary steroids, or no relationship (as is seen in the hypertensive control cohort) would be expected. Furthermore, this is the first study to demonstrate that levels of steroid excretion correlate with adverse end organ effects in CKD and that levels interact with sodium status, which supports the concept that blockade of the MR and reduction in dietary sodium intake should be considered in CKD.

5 Chapter Five - A study of the determinants of left ventricular abnormalities in patients with CKD

5.1 Introduction

Renal disease is associated with a variety of cardiovascular abnormalities leading to a graded increased cardiovascular risk, such that patients with CKD stage 5 are thirty times more likely to die a cardiovascular death than age matched controls(5). Left ventricular hypertrophy (LVH) is present in at least 50% of patients with CKD by the time they require renal replacement therapy (198) and LVH is an independent risk factor for mortality, both in patients with ESRD(288) and in the general population(289). Other LV abnormalities are also seen in patients close to or requiring RRT and are termed uraemic cardiomyopathy. This includes left ventricular hypertrophy (LVH), dilatation, systolic dysfunction (LVSD) and myocardial fibrosis(200).

However, left ventricular abnormalities are less well studied in patients with earlier stages of renal disease. Previous studies have utilised echocardiography(290) which is limited by its reliance on intravascular volume status or electrocardiography(291) which can be unreliable. In this study, the gold standard measurement of left ventricular mass, cardiac magnetic resonance imaging (CMR), was used to measure left ventricular mass and function in patients with CKD and patients with EH. By comparing the two groups, it was aimed to control for the effects of hypertension.

The aims of this study were:

1. To describe left ventricular abnormalities in patients with CKD and compare these with a group of patients with EH.

2. To assess the factors associated with LV abnormalities in CKD, particularly level of renal function, steroid status and sodium status; and to assess whether an interaction existed between these factors.

5.2 Methods

Patients were recruited and assessed according to the patient protocol in chapter 2. CMR scanning was performed as previously described and was undertaken at the same time as blood and urinary assessments. Cut-offs for left ventricular abnormalities were as described in Table 5-1. Statistics were performed using SPSS (Illinois) version 15.

Left ventricular abnormality	*Measure*	*Male*	*Female*
LVSD	LVEF	<55%	<55%
LVH	LVMI (LVM/BSA)	>84.1g/m^2	>76.4g/m^2
LV dilatation	EDV/BSA	>111.7ml/m^2	>99.3ml/m^2
LV dilatation	ESV/BSA	92.8ml/ m^2	70.3ml/ m^2

Table 5-1: Gender specific cut-offs for left ventricular abnormalities.

5.3 Results

5.3.1 Demographics

87 out of a potential 100 patients were included in the study. One patient was excluded due to having a permanent pacemaker. Nine patients were unable to complete the scan due to claustrophobia and three patients were unable to enter the scanner due to extreme obesity. Of included patients, 29 patients had essential hypertension (EH) and 58 had CKD (25 patients had diabetic nephropathy (DMN), 25 had IgA nephropathy (IGAN) and 8 had membranous nephropathy (MGN)). Baseline demographics are described in table 5-2.

Mean eGFR and haemoglobin were significantly lower in the CKD group than the EH group and median proteinuria and PWV significantly higher. Diastolic blood pressure was significantly higher in the EH group. No CKD patients had nephrotic syndrome, 1 patient was taking a phosphate binder, 1 patient was taking a vitamin D analogue and 5 were prescribed an erythropoiesis stimulating agent.

Within the CKD group, there were some differences in demographics between primary renal diseases. Patients with DMN had significantly lower eGFR and haemoglobin and significantly higher waist circumference, pulse wave velocity, SBP, pulse pressure and LVMI. Patients with IgAN had significantly less proteinuria excretion.

	EH (n=29)	*CKD (n=58)*	*EH vs. CKD*	*DMN (n=25)*	*IgAN (n=25)*	*MGN (n=8)*	*All groups (p value)*	*PRD*
Age (y)	55.7 (9.2)	57.9 (13.2)	NS	61.3 (14.1)	53.2 (12.3)	61.8 (9.0)	NS	NS
% Male	82.8%	72.4%	NS	72%	76%	38%	NS	NS
Waist circ (cm)	100.7 (12.3)	99.6 (12.5)	NS	106.1 (11.5)	95.2 (12.3)	93.8 (7.0)	**0.009**	**0.003**
BMI (Kg/m^2)	29.3 (4.9)	29.1 (5.2)	NS	30.9 (5.2)	28.0 (4.8)	27.0 (5.2)	NS	NS
% History of vascular disease	6.9	22.4	NS	36	12	12.5	NS	NS
% Current smokers	10.3	15.8	NS	8.3	16	37.5	NS	NS
SBP (mmHg)	151 (20)	147 (23)	NS	159 (18)	134 (18)	146 (30)	**<0.001**	**<0.001**
DBP (mmHg)	93 (11)	82 (11)	**<0.001**	80 (12)	83 (9)	82 (14)	**<0.001**	NS
PP (mmHg)	58 (14)	65 (20)	NS	79 (14)	51 (13)	64 (20)	**<0.001**	**<0.001**
No. Anti-BP	2.1 (1.0)	2.6 (1.4)	NS	2.8 (1.5)	2.4 (1.3)	2.4 (1.3)	NS	NS
% Prescribed ACE/ARB	75.9	84.5	NS	76.0	92	87.5	NS	NS
Hb (g/dl)	14.6 (1.4)	12.4 (1.6)	**<0.001**	11.6 (1.3)	13.2 (1.6)	12.5 (1.4)	**<0.001**	**0.001**
eGFR (ml/min/1.73m^2)	89.8 (18.2)	38.5 (22.5)	**<0.001**	29.5 (13.4)	42.5 (24.3)	54.5 (29.0)	**<0.001**	**0.01**
uPCR (mg/mmol)	0.0 (0.0-4.0)	62 (22.5-199)	**0.003**	120.5 (81.2-107.7)	33.2 (19.0-99.5)	90.5 (48-313)	**<0.001**	NS
QP (g/24h)	0.1 (0.1-0.1)	0.8 (0.3-2.6)	**<0.001**	1.5 (0.5-3.7)	0.5 (0.3-1.2)	1.2 (0.6-4.0)	**<0.001**	NS
USod (mmol/24h)	156.2 (81.1)	166.8 (72.5)	NS	180.5 (68.5)	147.9 (61.8)	179.9 (105.6)	NS	NS
PRC (uIU/ml)	35.4 (17.7-99.3)	69 (17-214)	NS	65.8 (18.5-221.5)	112.4 (36.3-395.2)	10.6 (2.5-80.7)	NS	NS
PAC (pmol/l)	238 (160-333)	249 (162-443)	NS	264 (152-416)	253 (182-521)	155 (125.8-460)	NS	NS
THALDO (mcg/24h)	62.5 (24.4)	55.0 (20.1)	NS	57.1 (20.1)	52.1 (21.9)	56.5 (15.4)	NS	NS
THDOC (mcg/24h)	75.7 (33.0)	65.4 (23.9)	NS	70.2 (24.4)	60.0 (23.5)	66.2 (22.7)	NS	NS
Cortisol metabolites (mcg/24h)	10005 (5068)	8620 (4641)	NS	7538 (3690)	10065 (5795)	8029 (2756)	NS	NS
PWV (m/s)	7.7 (6.7-9.2)	9.4 (7.0-13.1)	**0.005**	11.8 (9.6-15.1)	7.0 (5.6-9.5)	8.2 (6.2-10.7)	**<0.001**	**<0.001**

Table 5-2: Baseline demographics of the different cohort groups.
Data are mean (SD) or median (IQR). Comparisons of All groups (EH, DMN, IGAN, MGN) or by primary renal disease in patients with CKD (DMN, IGAN or MGN) undertaken by one-way ANOVA, Kruskall Wallis or Chi Square test as appropriate. EH vs. CKD compared by t-test, Mann Whitney U test or Chi-Square test. p<0.01 deemed as significant (highlighted in yellow).

5.3.2 Left ventricular abnormalities

Twenty eight patients with CKD had LVH (48.3%). Five patients out of 58 had LVSD (7.1%) and 2 patients (3.4%) had left ventricular dilatation (one of whom had LVSD).

Sixteen patients with EH had LVH (55.2%), including 2 patients with other cardiac abnormalities. Two out of 29 patients had LVSD (6.9%), and the same 2 patients had left ventricular dilatation.

There were no significant differences in frequency of LV abnormalities between patients with CKD and patients with EH, but within the CKD group, LVH was more common in patients with DMN (Table 5-3).

	EH *(n=29)*	*CKD* *(n=58)*	*EH vs. CKD*	*DMN* *(n=25)*	*IgAN* *(n=25)*	*MGN* *(n=8)*	*All groups (p value)*	*PRD*
LVMI (g/m^2)	86.1 (71.3-98.4)	81.2 (70.1-89.4)	NS	92.6 (81.2-107.7)	72.3 (63.4-83.2)	77.3 (66.4-101.8)	**0.009**	**0.004**
LVEF (%)	68.1 (10.5)	68.9 (10.9)	NS	70.9 (7.7)	67.1 (12.6)	68.6 (10.7)	NS	NS
EDV/BSA (ml/ m^2)	67.1 (15.6)	60.3 (16.1)	NS	57.8 (12.0)	62.4 (11.8)	61.8 (31.6)	NS	NS
ESV/BSA (ml/ m^2)	21.8 (8.6)	19.9 (10.3)	NS	16.9 (6.3)	21.3 (9.4)	24.5 (19.1)	NS	NS
LVH (n,%)	16, 55%	28, 48%	NS	19, 76%	6, 24%	3, 38%	**0.002**	**0.001**
LVSD (n,%)	2, 7%	5, 7%	NS	1, 4%	3, 12%	1, 13%	NS	NS
LV dilatation (n,%)	2, 7%	2, 3%	NS	0, 0%	1, 4%	1, 13%	NS	NS

Table 5-3: Left ventricular measurements and frequency of abnormality by patient group.
Data expressed as mean (SD), n (absolute number) or % (percentage of group). Comparisons by Student's t-test, one-way ANOVA, chi-square or Kruskall Wallis test as appropriate. Significant differences (p<0.01) highlighted in yellow.

5.3.3 Correlations between measures of cardiac structure and function and demographics

Due to the small number of patients with LVSD or LV dilatation, these abnormalities were not considered further. This study will focus on left ventricular mass.

5.3.3.1 Correlations with left ventricular mass index

The following demographics were entered into a correlation matrix with LVMI: Age, sex, smoking status, drug treatment (ACE, ARB, BBl, CCB, Alpha block, diuretic, aspirin, statin), waist circumference, BMI, SBP, DBP, PP, MAP, HR, eGFR, albumin, CRP, urate, total cholesterol, phosphate, PTH, vitamin D, Hb, 24h QP, uPCR, 24h USod, logPAC, √PRC, THALDO, cortisol metabolites, THDOC, urinary free cortisone (E), urinary free cortisol (F) excretion, E:F ratio, RHI, PWV and AIx@75bpm.

In patients with CKD, there was a significant correlation between LVMI and male gender, blood pressure (SBP, PP and MAP), urinary protein excretion (24h QP or uPCR), THALDO excretion, THDOC excretion and usage of alpha blockers (Table 5-4).

In patients with EH there was also a significant correlation between male gender and LVMI, as well as AIx@75bpm and PAC. There was no significant correlation with SBP or DBP.

Demographic	*All patients*	*EH*	*CKD*
Male gender	0.491, <0.001	0.491, 0.007	0.479, <0.001
Ablocker	0.357, 0.001		0.437, 0.001
SBP	0.462, <0.001		0.55, <0.001
PP	0.433, <0.001		0.491, <0.001
MAP	0.353, 0.001		0.459, <0.001
24h QP	0.323, 0.003		0.538, <0.001
PCR	0.293, 0.006		0.513, <0.001
AIx@75bpm		-0.486, 0.007	
Log PAC		0.57, 0.001	
THAldo			0.43, 0.001
THDOC			0.447, 0.001

Table 5-4: Significant correlations with LVMI in all patients, patients with EH and patients with CKD. ***Values are r,p. p<0.01 = significant.***

5.3.4 Specific associations with LVMI

5.3.4.1 Impact of the level of renal function on LV mass and function

Assessing all patients together, estimated glomerular filtration rate (eGFR), measured using the 6v-MDRD formula, was not associated with LVMI, EF, EDV adjusted for BSA (cEDV) or ESV adjusted for BSA (cESV)(Figure 5-1).

Comparing the proportion of patients with LVH by stage of CKD (2, 3 or 4), it can be seen that more patients were classified as having LVH in the stage 4 group (63%) compared with the stage 3 group (35%) or stage 2 (20%), although this failed to reach significance (one-way ANOVA p=0.08) (Figure 5-2).

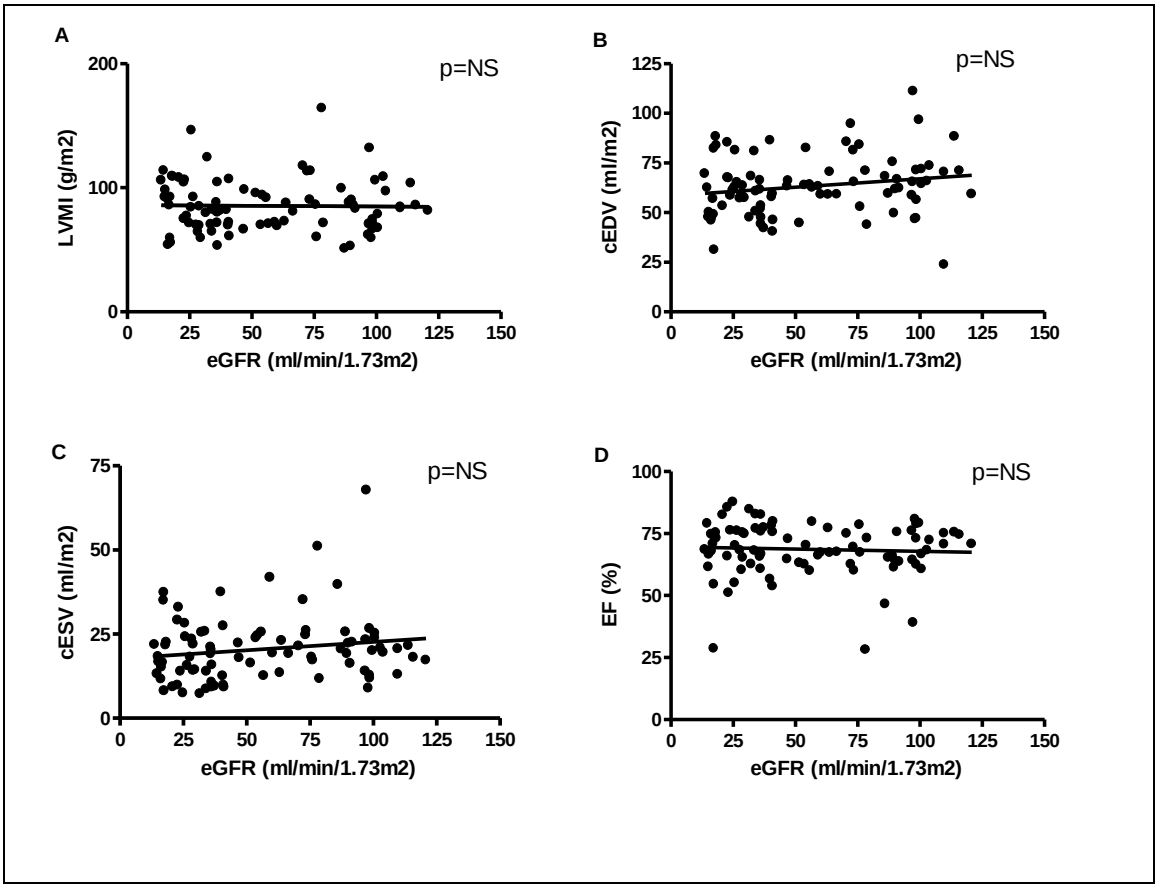


Figure 5-1: Scatterplot of estimated GFR (6v-MDRD formula) versus left ventricular measures.
A: Left ventricular mass index; B: End diastolic volume/BSA; C: End systolic volume/BSA; D: Ejection fraction. Fitted linear regression line and an estimate of significance (p). All patients (CKD and EH) were included in analysis.

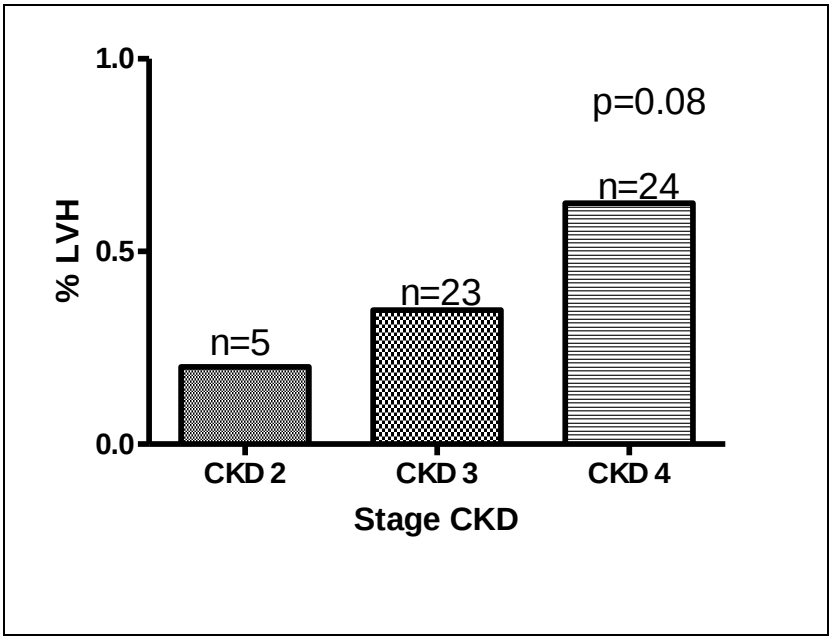


Figure 5-2: Bar chart of percentage of patients with LVH by stage of CKD

5.3.4.2 Association between plasma renin concentration, plasma aldosterone concentration and LVMI

Analysing EH patients, a higher PAC was associated with a significantly higher LVMI (Figure 5-3). However, there was no significant association between PRC and LVMI. Assessing CKD patients and different CKD groups, no significant relationship was seen between PAC, PRC and LVMI (Figure 5-3).

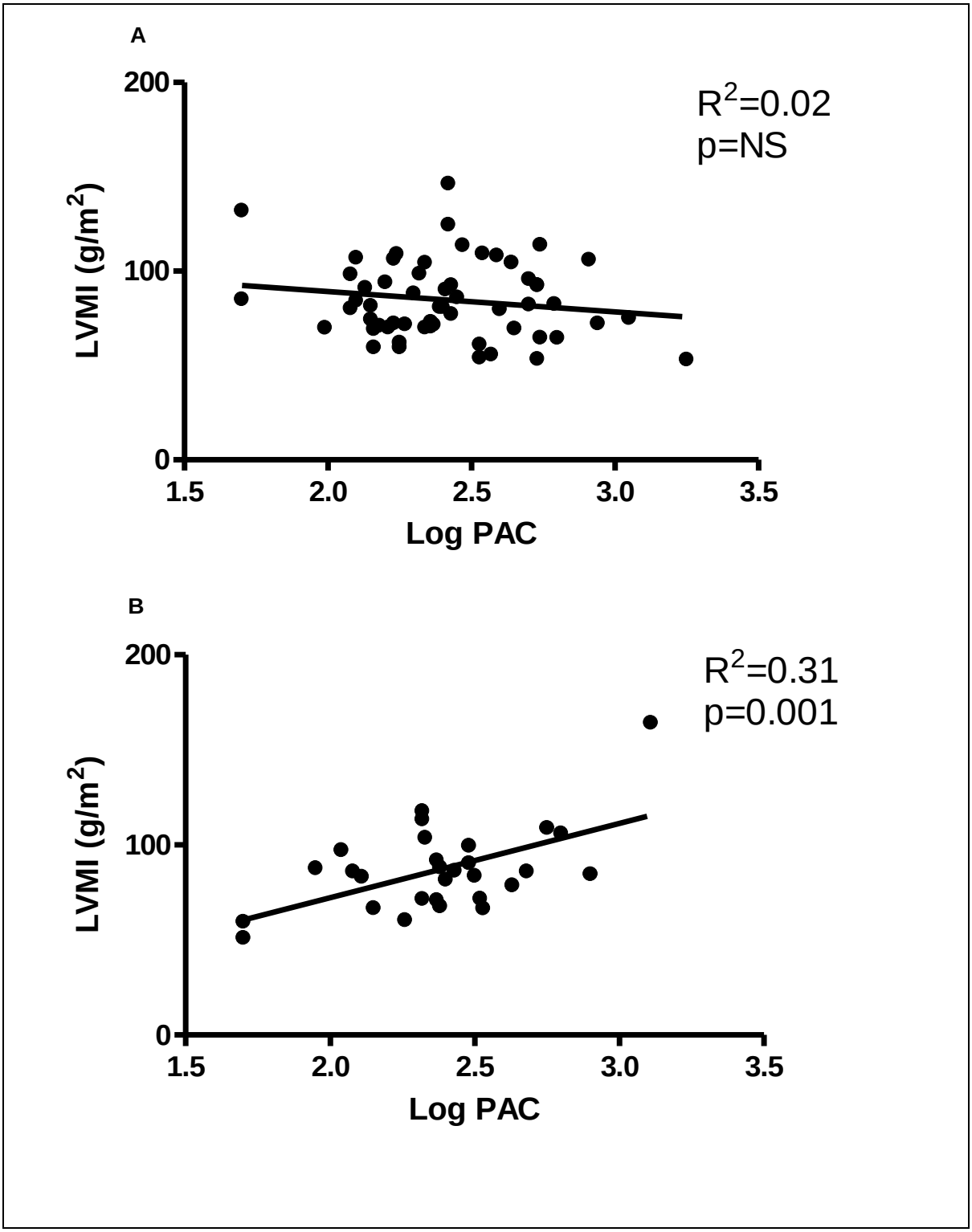


Figure 5-3: Scatterplot, with fitted linear regression line, of logPAC and LVMI in patients with CKD (A) and EH (B).

5.3.4.3 Association between proteinuria and LVMI

In patients with CKD, there was a significant correlation between the level of proteinuria and LVMI. This relationship persisted whether looking at the 24h urinary quantified protein or spot protein:creatinine ratio and persisted when looking at individual primary renal diseases (IGAN, DMN, MGN) (Figure 5-4).

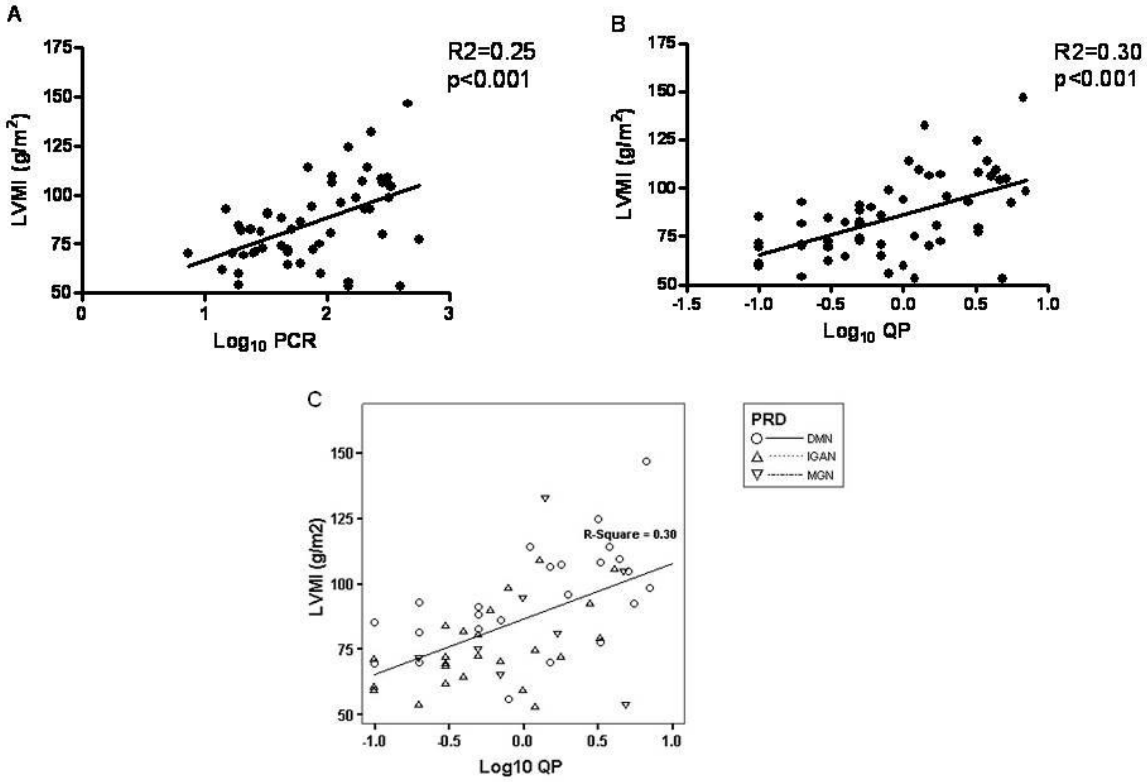


Figure 5-4: Scatterplots of logPCR (A) and log24h QP (B) versus LVMI in patients with CKD and subdivided by primary renal disease (C).
Fitted linear regression line and estimate of significance.

5.3.4.4 Association between urinary sodium excretion and LVMI

In CKD patients, urinary sodium excretion was a significant predictor of LVMI (p=0.03) but not in patients with EH. (Figure 5-5).

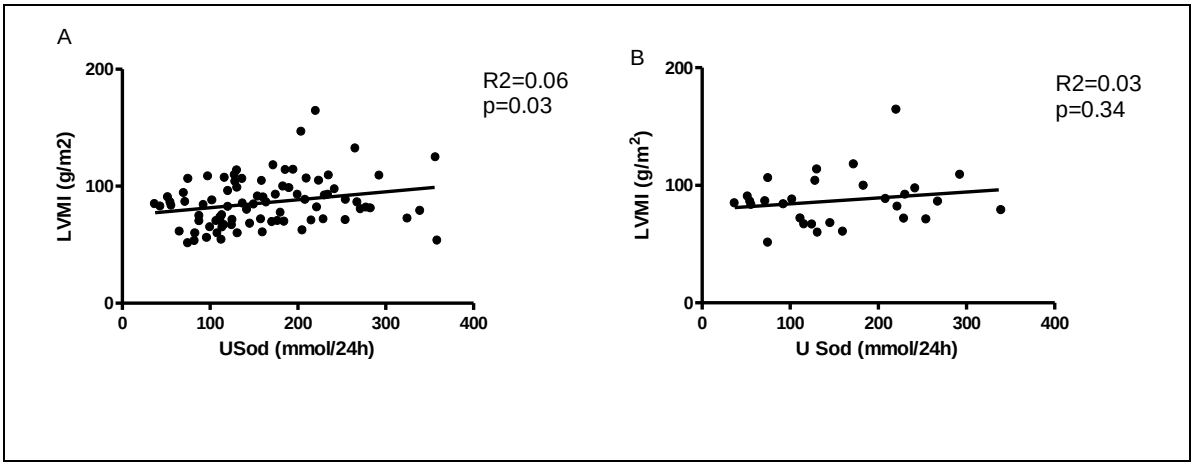


Figure 5-5: Scatterplot of urinary sodium excretion versus LVMI with fitted linear regression line. ***A = CKD, B = EH.***

5.3.4.5 Association between urinary steroid excretion and LVMI; interaction with urinary sodium

THAldo and THDOC excretion both correlated significantly with LVMI in patients with CKD (Figure 5-6). On linear regression analysis, both were also significant univariate predictors of LVMI (THAldo R^2 = 0.12 p= 0.009, THDOC R^2=0.16 p=0.002) in patients with CKD. This relationship was not seen in patients with EH (Figure 5-6).

In patients with CKD, to ascertain whether there was a synergistic relationship between THDOC excretion, urinary sodium (both significant univariate predictors) and LVMI, a 3D bar chart of tertiles was constructed (Figure 5-7). In the high THDOC group, increasing tertiles of USod were associated with in an incremental increase in LVMI. It can be seen that in the low THDOC group, higher USod is not associated with a higher LVMI. To ascertain whether there was a significant interaction between the effects of THDOC and USod on LVMI, two-way ANOVA was carried out based on group means, which revealed no significant interaction.

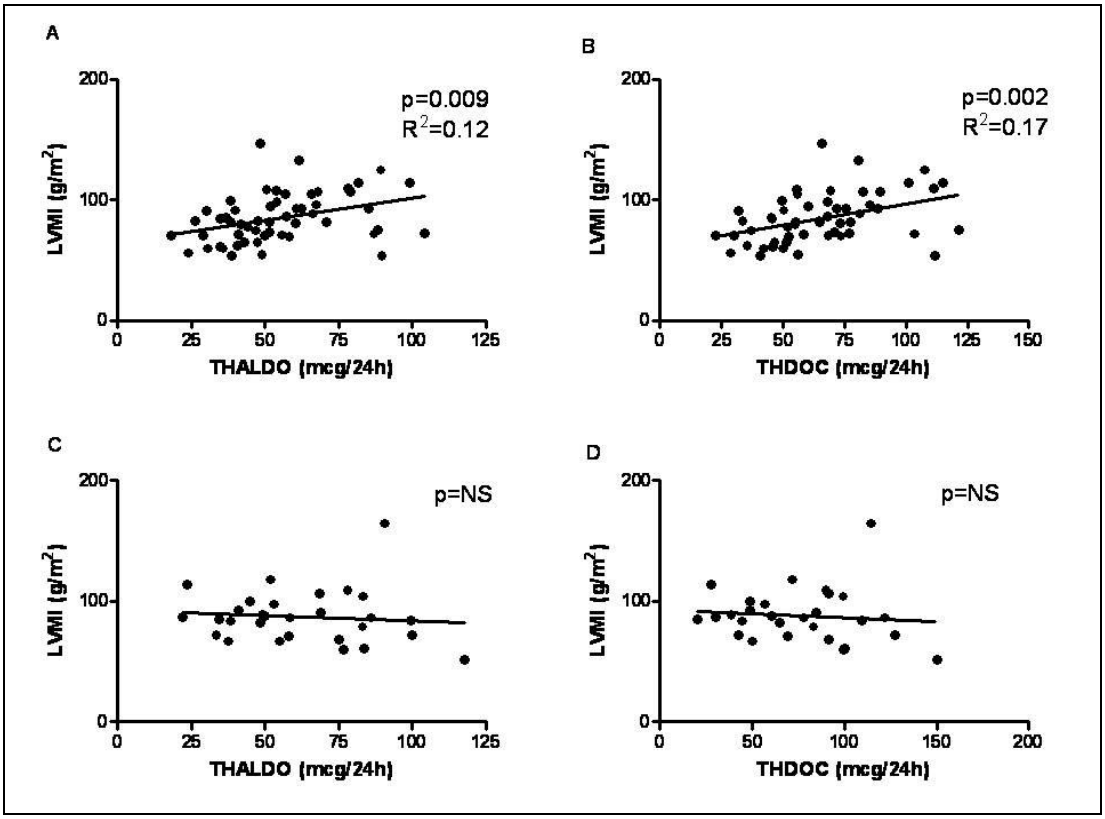


Figure 5-6: Scatterplots of THALDO (A&C) and THDOC (B&D) excretion versus LVMI with fitted linear regression line and estimates of significance.
A&B = CKD patients, C&D = EH patients.

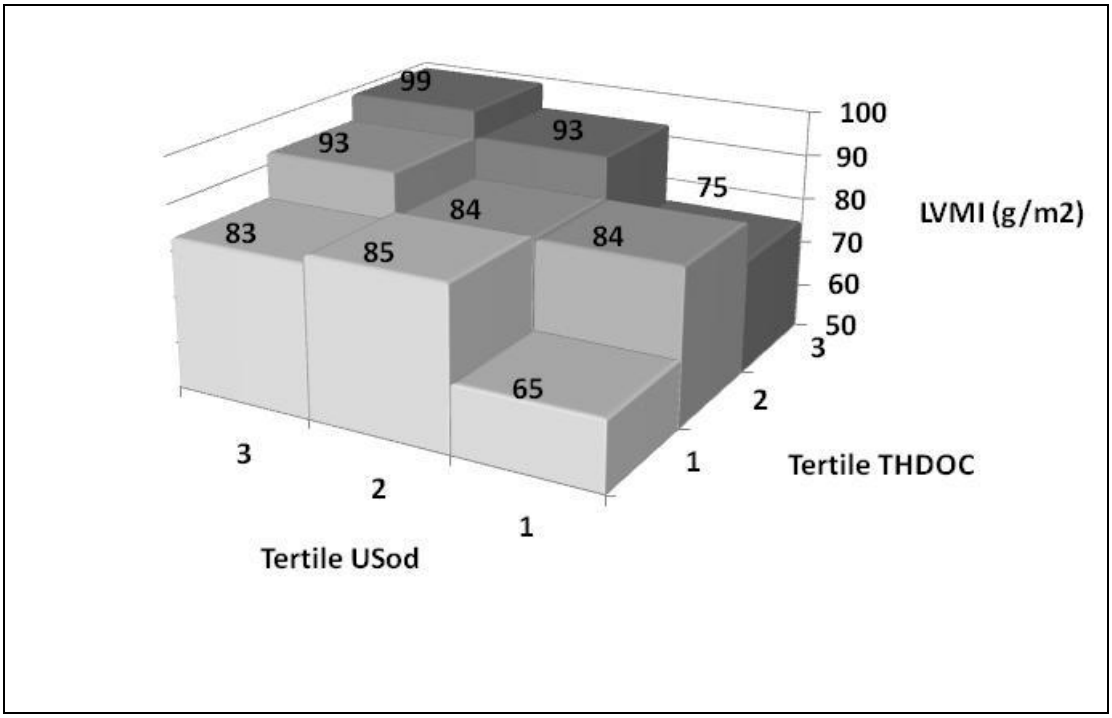


Figure 5-7: 3D bar chart of mean LVMI (g/m2) by tertile of THDOC excretion and tertile of 24h USod (1=low, 3=high).

Graph displays CKD patients only.

5.3.5 Determinants of LVMI in patients with CKD versus patients with EH

To determine whether significant predictors of LVMI in patients with CKD were different from patients with EH, significant correlates (Table 5-4) were entered individually into a univariate linear regression model, with the predicted variable of LVMI. Significant univariate predictors were then entered together into a multivariate model.

THAldo and THDOC excretion were entered separately into the models due to multicollinearity. Similarly, only the most closely correlated measure of proteinuria and blood pressure were entered into the model.

5.3.5.1 Patients with CKD

Significant univariate predictors of LVMI in patients with CKD were male gender, SBP, THAldo, THDOC excretion, 24h USod, usage of alpha blockers and 24h QP. Table 5-5 demonstrates the multivariate model when THAldo was included, demonstrating that THAldo was not a significant independent predictor of LVMI in patients with CKD but SBP, male gender and alpha blocker usage were. Table 5-6 shows the multivariate model when THDOC is included and it can be seen that THDOC excretion, SBP, male gender and usage of alpha blockers were significant independent predictors of LVMI in patients with CKD on multivariate analysis. Quantified proteinuria was no longer a significant independent predictor of LVMI when THDOC or THAldo excretions were included in the model. The addition of eGFR to the model did not alter the findings.

Variable	*Univariate* *B*	*95% CI*	*β*	*P*	*Multivariate Model 1* *B*	*95% CI*	*B*	*p*
THALDO (mcg/24h)	0.002	0.0-0.03	0.352	0.009	-	-	-	-
SBP (mmHg)	0.002	0.001-0.003	0.546	<0.001	0.002	0.001-0.003	0.381	<0.001
24h USod (mmol/24h)	0.00	0.00-0.001	0.271	0.045	-	-	-	-
Male gender	0.113	0.06-0.165	0.497	<0.001	0.075	0.0.32-0.117	0.315	0.001
Log 24h QP	0.028	0.014-0.041	0.491	<0.001	-	-	-	-
Alpha blockade	0.123	0.059-0.187	0.458	<0.001	0.083	0.036-0.13	0.31	0.001

Table 5-5: Univariate and multivariate model of predictors of LVMI in patients with CKD when THAldo excretion analysed in model.
Predicted variable logLVMI. R^2=0.662.

Variable	*Univariate* *B*	*95% CI*	*B*	*P*	*Multivariate Model 2* *B*	*95% CI*	*β*	*P*
THDOC (mcg/24h)	0.002	0.001-0.003	0.404	0.002	0.001	0.0-0.002	0.218	0.02
SBP (mmHg)	0.002	0.001-0.003	0.546	<0.001	0.002	0.001-0.003	0.384	<0.001
24h USod (mmol/24h)	0.00	0.00-0.001	0.271	0.045	-	-	-	-
Male gender	0.113	0.06-0.165	0.497	<0.001	0.074	0.033-0.114	0.312	0.001
Log 24h QP	0.028	0.014-0.041	0.491	<0.001	-	-	-	-
Alpha blockade	0.123	0.059-0.187	0.458	<0.001	0.08	0.034-0.125	0.297	0.001

Table 5-6: Univariate and multivariate model of predictors of LVMI in patients with CKD when THDOC excretion analysed in model.
Predicted variable log LVMI. R^2=0.683.

Variable	*Univariate* *B*	*95% CI*	*B*	*P*	*Multivariate Model 2* *B*	*95% CI*	*β*	*P*
Log PAC	0.189	0.081-0.297	0.57	0.001	0.147	0.026-0.268	0.442	0.02
AIx@75bpm	-0.005	-0.001- -0.008	-0.448	0.015	-	-	-	-
Male gender	0.138	0.045-0.232	0.506	0.005	-	-	-	-

Table 5-7: Univariate and multivariate model of predictors of LVMI in patients with EH.
Predicted variable log LVMI. R^2=0.444 for multivariate model.

Patients with EH

Table 5-7 demonstrates the multivariate model for predictors of LVMI in patients with EH. It can be seen that PAC is the only significant independent predictor of LVMI in these patients.

5.3.5.2 Differences between CKD and EH group predictors of LVMI

It can therefore be seen that the predictors of LVMI differ between patients with EH and patients with CKD. Male gender is a significant univariate predictor in both groups but not a multivariate predictor in EH. In CKD, SBP, THDOC excretion and alpha blockade are significant multivariate predictors, which differs from EH patients where only PAC is a significant multivariate predictor of LVMI.

5.4 Discussion

This study of LV abnormalities in patients with CKD and patients with EH utilises volume-independent cardiac MR imaging. The groups were well matched in terms of baseline demographics, although patients with EH had a significantly higher DBP. The CKD group had lower levels of renal function and higher levels of proteinuria.

The key findings were:

1. Around 50% of patients had evidence of left ventricular hypertrophy in both the EH and CKD groups.

2. Level of renal function is not directly associated with left ventricular mass.

3. Excretion of THDOC, in addition to gender, SBP and alpha blocker usage, is a significant independent predictor of LVMI in patients with CKD but not patients with EH.

4. An inter-relationship between higher levels of urinary sodium excretion, higher levels of THDOC excretion and higher LVMI was seen in patients with CKD.

5. In patients with EH, plasma aldosterone concentration is a significant independent predictor of LVMI.

Identification of the differing predictors of LVMI is of importance if we are to unravel the processes leading to the excess cardiovascular risk associated with LVH in both patients with CKD and patients with EH.

5.4.1 Frequency of cardiac abnormalities in patients with CKD

The predominant cardiac abnormality seen in patients with CKD was LVH, present in 48% of patients in this cohort. LVSD and LV dilatation were relatively infrequent. A comparable study of patients with ESRD assessed using CMR demonstrated that 71.6% of patients had LVH, 11.2% had LV dilatation and 8.2% had LVSD(200).

Edwards et al(131) also utilised CMR to assess patients with non-diabetic minimally proteinuric CKD 2 or 3 (mean eGFR 53ml/min/m2) and reported a mean LVMI of 59.2g/m^2 and a prevalence of LVH of only 8%. This is significantly lower than seen in our cohort. However, this is likely due to differences in the study populations, including better blood pressure control, better preserved renal function and the lack of diabetic patients in that study.

The higher LVMI seen in patients with DMN than in patients with primary glomerular disease is similar to findings by Nardi et al(292), where the higher LVMI in diabetic patients was reported to be independent of other factors including blood pressure. It would have been useful to have a comparator group with diabetes without nephropathy in this study.

Levin et al(293) found LVH by echocardiography to be present in 39% of patients with CKD, with the incidence increasing with declining eGFR. Nardi et al found a prevalence of LVH of 55.9% by echocardiography in patients with CKD, and found the ejection fraction to be well preserved. In a substudy of the CREATE study, prevalence of LVH (by echo) was 47% and significantly associated with cardiovascular risk(294).

The lack of a significant difference in the prevalence of LVH reported using echo and CMR suggests that the inaccuracies associated with assessing chamber size using echo are perhaps less important in CKD than in ESRD, where volume overload is more of an issue.

Using ECG Cornell criteria for LVH, Agarwal et al(291) found a prevalence of LVH of only 11% in patients with CKD. This is in keeping with findings in ESRD whereby the frequency of LVH reported by ECG is lower than other measures (295), suggesting that

this method grossly underestimates the prevalence of LVH, making it of limited value for this purpose(197).

5.4.2 Factors associated with left ventricular mass index in CKD

5.4.2.1 LVMI and blood pressure

The association of LV mass with systolic blood pressure seen in the CKD cohort is as would be expected in terms of increased preload and afterload. Volume overload results in myocardial stretch and proliferation, with eccentric remodelling. An increased systemic pressure requires increased strength of ventricular contraction to maintain stroke volume, enabled by concentric remodelling.

Of interest, the lack of association of LVMI with carotid-femoral pulse wave velocity raises uncertainty about the role of increased arterial stiffness as a cause of increased afterload in CKD; this contrasts with the clear association in ESRD (202). The association of LVMI with pulse pressure suggests that perhaps fluid overload is of more importance.

The use of alpha blockade was associated with a higher LVMI. This agent is most often resorted to in patients with difficult to control blood pressure, as a third or fourth line agent and therefore blood pressure may be the real determinant here. Patients prescribed an alpha blocker had a significantly higher pulse pressure ($p=0.007$).

5.4.2.2 LVMI and level of renal function

LVH was present in half of our patients with moderately impaired renal function, and LVH increased with stage of CKD, albeit non-significantly. Estimated glomerular filtration rate was not significantly associated with myocardial mass. Studies using echocardiography (198;203) have found a step-wise increase in the prevalence of left ventricular hypertrophy with declining renal function (CKD stages 1-4). The MESA study (296) looked at the association of LVMI, using CMR, and renal function in patients with primary atherosclerotic disease with concomitant reduced eGFR and found a non-linear association with renal function, whereby LVH was associated with a GFR between 60 and 75 ml/min, but this was lost above 75ml/min and below 60ml/min, once adjustment was made for blood pressure. It is clear that LV mass has increased significantly by the time a patient starts dialysis(200) and it may be that changes in the left ventricle occur early in the course

of renal disease, in association with the development of hypertension and mild fluid overload. Further significant changes occur as renal function further deteriorates, in the pre-dialysis phase, in association with disturbances in bone metabolism and other uraemic factors(201).

5.4.2.3 LVMI and proteinuria

The association between proteinuria excretion and LVMI on univariate analysis in CKD is intriguing, but when included in multivariate analysis with measures of urinary steroid excretion, this finding was no longer significant. The role of proteinuria in determining LV mass has been explored by other investigators, but not with access to measures of steroid metabolism.

5.4.2.4 LVMI and sodium

In this study a higher urinary sodium was associated with a higher LVMI in patients with CKD. Urinary sodium excretion was not, however, a significant multivariate predictor of LVMI. The mechanism of the association of sodium status and LVMI is likely to include volume overload resulting in myocardial stretch. Also, increasing plasma sodium levels can impair the release of vasodilatory nitric oxide in the circulation(297), increasing systemic pressure. Additionally, salt sensitive hypertensive patients on a high sodium intake demonstrate increased sympathetic tone (increased urinary catecholamines) and enhanced pressure response to AgII, potentially favouring the onset of LVH(281).

In patients with EH, no association was found between urinary sodium excretion and LVMI. The Framingham Heart Study(215) examined the relationship between urinary sodium excretion (normalised to creatinine from a spot sample) and LVMI (measured using echocardiography) in Framingham offspring and found no association between the two measures in normotensive or hypertensive individuals. Utilising a spot sample is however flawed as it assumes that urinary sodium excretion is constant throughout the day.

5.4.2.5 LVMI and deoxycorticosterone (DOC)

DOC is a largely overlooked mineralocorticoid. It is produced in the zona fasciculata of the adrenal cortex, as well as extra-adrenally by 21-hydroxylation of plasma-borne progesterone. Its levels do not depend upon aspects of electrolyte status but upon ACTH

levels. It has an affinity similar to aldosterone for binding the mineralocorticoid receptor but is a weaker mineralocorticoid with a lower sodium retaining ability. There is, however, experimental and clinical evidence that DOC can have marked potency.

Administration of DOCA to uninephrectomized rats receiving a high sodium diet resulted in left ventricular hypertrophy and increased perivascular collagen(139). Rats treated with ALDO similarly developed LVH but the distribution of collagen differed. These results were replicated by others who also demonstrated that these effects could be prevented or reversed by the addition of eplerenone(143;298). In humans, deficiency of 11β-hydroxylase (in a rare form of congenital adrenal hyperplasia) results in elevated circulating DOC concentrations leading to hypertension in the majority of patients. In vivo work therefore suggests that DOC is a potential factor in the increase of cardiac mass in the context of a high sodium environment via collagen deposition and myocyte hypertrophy. However, its role in LVH in humans has not been examined prior to this study.

Ligand specificity of the mineralocorticoid receptor in the distal tubule is maintained by 11beta- hydroxysteroid dehydrogenase type 2 (11BHSD2), converting active cortisol (F) to inactive cortisone (E), leaving the MR free to bind with aldosterone. Recently, a similar enzyme has been identified which protects the MR from DOC binding(91). The aldo-keto reductase enzyme AKR1C3 converts DOC to inactive 20α-hydroxy-DOC in renal cortical and medullary collecting ducts, impairing activation of the MR. The effective concentration required to induce a 50% effect (Ec50) of DOC increased 1000-fold in presence of AKR1C3. These findings remain to be confirmed in vitro, particularly as the Km of the enzyme is high (uM) in relation to circulating concentrations of DOC (pM).

If, like 11BHSD2, the AKR1C3 enzyme is not expressed in the heart, it may leave DOC free to bind the MR, explaining the independent effect of DOC on left ventricular mass.

This novel finding of an association between DOC and LVMI merits further study.

5.4.2.6 LVMI and aldosterone

LVH is more frequent and severe in patients with primary hyperaldosteronism than in patients with EH(276). Associations between Aldo and LV mass have been reported in various subgroups, predominantly with hypertension. The following studies utilised echocardiography.

Utilising the Framingham cohort, Vasan et al(299) demonstrated that PAC was independently associated LV wall thickness, but only in women. Similarly, in the MONICA cohort (300) PAC was correlated with LVMI in hypertensive women, but not normotensive women or men.

Mule et al compared PAC and echo-determined LV mass in Caucasian hypertensives with and without the metabolic syndrome(301). Patients with the metabolic syndrome had a higher baseline PAC (due to obesity on multivariate analysis), higher SBP and LV mass. In both groups, PAC was significantly associated with LV mass (adjusted for height) but in the group with metabolic syndrome, PAC was independently associated with LV mass after adjustment for blood pressure, gender and BMI. This relationship was much weaker in patients without the metabolic syndrome.

El-Gharbawy et al(302) found PAC to be associated with LVMI, but only in black hypertensive patients with a BMI >27. Malmqvist et al(303) reported PRA was an independent predictor of LVMI in hypertensive patients with LVH, but not PAC. In a small study, Duprez et al(210) demonstrated that PAC was significantly associated with LVMI in patients with hypertension, even after adjustment for BP. Utilising urinary aldosterone measurement, Delles et al(304) assessed healthy Caucasian men and found urinary aldosterone to be associated with LV mass, but this was not subject to adjustment for body surface area and therefore was not left ventricular mass index. Contrary to this, Schlaich et al(305) assessed 76 healthy white men and found that baseline urinary aldosterone levels were not associated with LV structure or function.

In this current comparison of patients with EH, PAC was a significant independent predictor of LVMI, which supports the studies documented above and expands the findings to include males and findings were independent of BMI. Due to small numbers of females with EH in the study (n=5), subgroup analysis was not performed.

In patients with ESRD on haemodialysis, Sato et al(306) assessed the relationship between PAC and LVMI and found a significant association in non-diabetic patients but not in diabetics, although numbers were small.

There have been no previous studies assessing the relationship between Aldo and LVMI in patients with CKD. Our findings that Aldo was not significantly associated with LVMI but that THDOC was a significant independent predictor, suggests that different mechanisms

of increasing left ventricular mass may be seen in CKD as compared with EH. DOC is largely produced in response to ACTH, unlike aldosterone which responds to changes in electrolyte and fluid status. Despite this, there was no association between cortisol metabolites and LVMI in CKD, suggesting that ACTH is not the primary driver of this finding. An alternative explanation is that DOC binds and activates the cardiac MR in patients with CKD, but not EH, leading to myocyte hypertrophy and LVH. Why DOC should preferentially bind the MR over the traditional ligands of aldosterone or cortisol is unclear.

Inhibition of the MR is, however, the only pharmaceutical intervention demonstrated to regress LVH in patients with CKD(131). Edwards et al demonstrated that in 112 patients with non-diabetic CKD 3, randomised to spironolactone 25mg or placebo for 40 weeks in addition to ACE or ARB, spironolactone significantly reduced LV mass independent of reductions in SBP. Similar findings were seen in patients with EH in a separate study(307). Blockade of the MR is not, however, ligand specific and it may be that the beneficial effects seen may relate to blockade of DOC, not aldosterone (or cortisol in tissues devoid of 11βHSD2) as previously presumed.

No association was seen between a polymorphism of the aldosterone synthase gene (-344C/T) and LVMI, which mirrors previous findings(308).

5.4.2.7 LVMI and cortisol

A high prevalence of LVH is reported in patients with Cushing's syndrome(211). In a small group of patients with EH, urinary free cortisol and cortisone correlated significantly with LVMI, but not in controls. No adjustment was made for blood pressure or other potential correlates(309). In this study, no association was seen between cortisol metabolites, urinary free cortisol (F), urinary free cortisone (E) and LVMI in patients with CKD or patients with EH.

5.4.3 Interaction between steroids and sodium

Experimental data from animal models suggests a high sodium environment has a permissive effect on mineralocorticoids in terms of the development of ventricular

hypertrophy and fibrosis(139;141). In this study there was a non-significant trend towards an association between high urinary sodium excretion, high urinary steroid excretion and high LVMI. Larger numbers may be required to demonstrate an association.

The interaction between sodium, aldosterone and LV mass was studied in a hypertensive Belgian population(281) using echocardiography and urinary aldosterone excretion (from a 24h sample, measured using RIA). LVMI independently increased with the 24-hour urinary excretion of both sodium and aldosterone.

The mechanism by which a high sodium intake promotes mineralocorticoid mediated damage is uncertain but may relate to increased oxidative stress resulting in increased activation of the MR.

5.4.4 LVMI in patients with EH

The prevalence of LVH in the cohort of patients with EH is higher than would be expected for a general hypertensive cohort, where a prevalence of 15-20% by echo would be expected(310). It may be that the patients examined were not representative of a typical hypertensive cohort as they were recruited from a hospital blood pressure clinic, where, by definition, they have difficult to control blood pressure and might be more prone to developing end organ complications. The strong predictive nature of plasma aldosterone on LVMI differs from that seen in the CKD cohort but is in keeping with published data.

5.4.5 Limitations

As a cross-sectional analysis, this study cannot assume causality. Using estimated GFR rather than direct measurement will introduce some inaccuracy, although the MDRD6 formula is well validated in patients with CKD. There are baseline differences between the different groups of renal diseases, reflecting their primary medical conditions, but the baseline differences are unlikely to have influenced the study findings. Small patient numbers limit individual disease subgroup analysis and therefore differences between diseases must be interpreted with caution as they may reflect under-powering of the study or differences between EH, CKD and different renal diseases. Direct measurement of albumin excretion was not available in this cohort and would have been interesting to compare with total proteinuria, as one would expect albuminuria to be more closely correlated if the association with LVMI was mediated by endothelial dysfunction. It would

also be interesting to expand the study to include patients with milder CKD as increases in LVMI have been seen in patients with preserved renal function and biopsy proven GN(311) or APKD(312). Including larger numbers of patients would increase confidence in the existence of a relationship between steroids, sodium and LVMI in patients with CKD.

5.5 Conclusion

Although the identified predictors of LVMI are largely traditional and account for 68% of the variation in LVMI in patients with CKD, it remains unexplained why DOC should predict LVMI in patients with chronic kidney disease. This finding is not seen in patients with EH and suggests that DOC and possibly MR binding may have a role in explaining the excess cardiovascular morbidity and mortality seen in patients with CKD, independent of the effects of blood pressure.

6 Chapter Six: A study of the association between mineralocorticoids, sodium and vascular function in patients with chronic kidney disease

6.1 Introduction

In patients with CKD and ESRD, pulse wave velocity is independently associated with an increased risk of renal progression(242) and death(235;243). The main determinants of vascular function in patients with CKD are blood pressure and age however these do not fully explain the increased vascular stiffness seen. Endothelial function is also dysregulated in CKD(313) and may contribute to the increased vascular risk seen in these patients.

Spironolactone treatment was shown to reduce vascular stiffness in patients with CKD(131), however whether levels of mineralocorticoids and sodium intake are directly associated with large and small vessel function in CKD has not been studied.

The aims of this study were:

1. To assess which factors were associated with vascular stiffness and endothelial function in CKD, and to compare this with patients with EH.

2. To determine whether steroid status impacts upon vascular and endothelial function in patients with CKD and patients with EH, and whether sodium has a modulatory role in this situation.

6.2 Methods

Patients with CKD and hypertensive controls were recruited and the augmentation index (AIx), carotid-femoral pulse wave velocity (PWV) and endothelial reactive hyperaemia index (RHI) were assessed according to the protocol in chapter 2. The AIx was normalised to a heart rate of 75bpm due to the close correlation between AIx and heart rate – AIx@75bpm. Unless specified, this is the measure which will be assessed.

6.2.1 Statistical analysis

Differences between groups were compared using Student's T-test, Mann Whitney U test, one-way ANOVA or Kruskall Wallis test as appropriate. Pearson's and Spearman's correlation coefficients were calculated for normally and non-normally distributed data, respectively. $P \leq 0.01$ was deemed significant due to multiple calculations. PWV and RHI

were logarithmically transformed to achieve normality. Linear regression was used to determine which significant correlates were significant univariate predictors of the dependent variable. Significant univariate predictors were then were entered into multivariate linear regression models.

6.3 Results

6.3.1 Patient demographics

Patient demographics are described in detail in chapter 4 (Table 4-1). Briefly, the mean age was 57.4 (SD 11.9) years, 78% of patients were male, mean SBP was 149 (SD 22) mmHg and mean DBP was 86 (SD 13) mmHg and mean BMI was 29.3 (SD 5.5) g/m2. Mean calculated augmentation index was 22.8 (SD 10.3) %, median PWV 8.9 (7.0-11.6) m/s and median RHI 1.8 (1.7-2.4).

Within the CKD cohort, patients with IgAN were significantly younger and had significantly lower SBP. Diabetic patients had significantly lower levels of renal function.

Drug therapies were varied across the whole cohort (Table 4-6). EH patients were significantly more likely to be prescribed thiazide diuretics (56% vs. 6%) and significantly less likely to be prescribed a statin (43% vs. 69%) or ACEi (37% vs. 64%).

Biochemical differences between the hypertensive and CKD cohorts are as would be expected. Renal patients had significantly lower eGFR, higher urate, higher CRP, lower serum albumin, higher phosphate, higher PTH, lower vitamin D, lower haemoglobin and had higher levels of proteinuria. They also had significantly lower levels of urinary potassium excretion (tables 4-2 and 4-3).

Mean AIx@75bpm was 22.8 (SD 10.3), median PWV 8.9 (IQR 7.0-11.6) m/s and median RHI 1.8 (1.7-2.4). Pulse wave velocity was significantly higher in the CKD group than the EH group. There was no significant difference between groups in terms of augmentation index or reactive hyperaemia index measurements. Within the CKD group, patients with DMN had a significantly higher PWV (table 6-1).

Variable	EH controls N=30	CKD patients N=70	p	DMN N=34	IgAN N=27	MGN N=9	p
AIx	26.4 (11.6)	23.7 (10.3)	NS	24.6 (10.7)	21.1 (9.7)	27.9 (10.1)	NS
AIx@75bpm	24.9 (10.8)	22.1 (10.1)	NS	23.7 (10.0)	19.1 (10.4)	24.7 (8.8)	NS
PWV (m/s)	**7.9 (6.7-9.1)**	**9.4 (7.3-13.5)**	**0.006**	**11.8 (9.3-15.3)**	**7.0 (5.8-9.4)**	**7.7 (6.3-10.3)**	**<0.001**
RHI	1.9 (1.7-2.4)	1.8 (1.5-2.4)	NS	1.6 (1.4-2.2)	2.0 (1.6-2.5)	1.8 (1.5-2.7)	NS

Table 6-1: Baseline vascular function, stratified by group.
Data expressed as mean (SD) or median (IQR). Comparison by t-test, Mann Whitney U test, one-way ANOVA or Kruskall Wallis test as appropriate.

6.3.2 Correlations between markers of vessel function and potential determinants

Table 6-2 illustrates the correlation matrix between vascular function and demographics, prescribed medications, plasma and urine variables and left ventricular findings in patients with chronic kidney disease. Table 6-3 shows the same correlation matrix in patients with essential hypertension.

Table 6-2: Correlation matrix in renal patients.

Pearson's or Spearman's correlation coefficient as appropriate. P≤0.01 deemed significant (highlighted in bold).

	Log RHI ***r, p***	***AIx*** ***r,p***	***AIx@75bpm*** ***r,p***	***Log PWV*** ***r,p***
Age	-0.289, 0.02	**0.305, 0.01**	0.262, 0.03	**0.602, <0.001**
Sex (male=0)	0.174, 0.15	0.29, 0.02	**0.4, <0.001**	-0.087, 0.50
Weight	-0.187, 0.12	**-0.312, 0.01**	**-0.481, <0.001**	0.141, 0.27
BMI	-0.199, 0.12	-0.064, 0.66	-0.171, 0.24	0.158, 0.28
Height	-0.077, 0.52	**-0.302, 0.01**	**-0.355, 0.003**	0.014, 0.92
Waist circumference	-0.222, 0.04	**-0.296, 0.007**	**-0.389, <0.001**	**0.286, 0.008**
IHD	-0.064, 0.53	0.119, 0.27	-0.033, 0.76	0.231, 0.03
ECG abnormality	-0.174, 0.09	0.088, 0.42	0.056, 0.61	**0.286, 0.007**
SBP	0.232, 0.05	**0.396, 0.001**	**0.407, 0.001**	**0.373, 0.002**
DBP	**0.336, 0.004**	0.002, 0.99	0.127, 0.31	-0.127, 0.317
PP	0.07, 0.57	**0.475, <0.001**	**0.411, 0.001**	**0.516, <0.001**
Drugs (1=yes) **-ACEi** **-ARB** **-BBlocker** **-Diuretic (loop)** **-Statin**	 0.014, 0.91 0.188, 0.12 0.205, 0.09 -0.054, 0.66 -0.143, 0.17	 -0.26, 0.03 0.03, 0.81 0.197, 0.11 -0.15, 0.22 -0.011, 0.92	 -0.121, 0.33 -0.07, 0.58 0.009, 0.94 -0.159, 0.20 -0.128, 0.24	 0.121, 0.34 -0.051, 0.69 0.167, 0.19 0.139, 0.28 0.273, 0.03
Log RHI	-	0.074, 0.55	0.128, 0.31	-0.234, 0.07
AIx	0.074, 0.56	-	0.832, <0.001	0.199, 0.12
AIx@75bpm	0.128, 0.31	0.832, <0.001	-	0.284, 0.03
Log PWV	-0.234, 0.07	0.199, 0.12	0.284, 0.03	-
EF	-0.003, 0.98	0.078, 0.57	-0.012, 0.93	0.086, 0.54
LVMI	0.03, 0.83	0.134, 0.32	0.09, 0.51	0.273, 0.05
EDV	0.121, 0.37	-0.096, 0.48	-0.099, 0.47	-0.113, 0.42
ESV	0.009, 0.95	-0.032, 0.82	-0.02, 0.88	-0.175, 0.21
eGFR (6v)	0.133, 0.27	0.044, 0.72	-0.008, 0.95	**-0.468, <0.001**
Cholesterol	0.13, 0.28	0.001, 0.99	0.024, 0.85	-0.298, 0.02
Cholesterol/HDL	-0.046, 0.71	0.005, 0.97	-0.095, 0.44	-0.02, 0.88
PTH	-0.112, 0.37	0.025, 0.85	0.01, 0.94	0.251, 0.05
PO4	-0.145, 0.24	0.126, 0.31	0.157, 0.21	0.297, 0.02
CRP	0.151, 0.22	0.001, 0.99	0.215, 0.09	0.03, 0.819
Hb	-0.011, 0.93	-0.134, 0.27	-0.273, 0.03	**-0.337, 0.007**
Vit D	0.107, 0.38	0.187, 0.13	0.152, 0.22	-0.177, 0.16
Glucose	-0.167, 0.18	0.306, 0.02	0.237, 0.06	**0.347, 0.006**
Urate	-0.029, 0.82	-0.149, 0.12	-0.201, 0.12	0.12, 0.36
PCR	-0.037, 0.76	0.139, 0.27	0.004, 0.97	0.197, 0.12
Log24h QP	-0.034, 0.78	-0.173, 0.16	-0.116, 0.36	0.194, 0.13
24h USod	**-0.349, 0.004**	-0.077, 0.49	-0.162, 0.14	0.221, 0.04
24h USod/U Pot	-0.218, 0.08	-0.036, 0.78	0.007, 0.96	**0.37, 0.003**
THALDO	-0.222, 0.08	-0.225, 0.08	-0.245, 0.06	-0.03, 0.82
THDOC	-0.228, 0.07	-0.201, 0.12	-0.165, 0.2	0.015, 0.91
Cortisol metabs	0.005, 0.97	-0.29, 0.02	**-0.388, 0.002**	-0.082, 0.53
Log Plasma aldo	-0.131, 0.29	-0.101, 0.42	-0.123, 0.33	-0.139, 0.27
PRC	-0.245, 0.04	**-0.462, <0.001**	**-0.389, 0.001**	-0.149, 0.24
ARR	0.18, 0.14	**0.366, 0.002**	0.291, 0.02	0.073, 0.56

	Log RHI *r, p*	*AIx* *r, p*	*AIx@75bpm* *r, p*	*Log PWV* *r, p*
Age	-0.084, 0.67	0.403, 0.06	0.343, 0.10	-0.03, 0.89
Sex (male=0)	0.435, 0.02	0.291, 0.18	**0.624, 0.001**	-0.118, 0.53
Weight	-0.216, 0.26	-0.223, 0.31	-0.331, 0.11	0.07, 0.71
BMI	-0.294, 0.12	-0.007, 0.97	0.107, 0.62	0.067, 0.72
Height	0.096, 0.62	-0.452, 0.03	**-0.715, <0.001**	-0.003, 0.99
Waist circumference	-0.145, 0.46	-0.124, 0.57	-0.172, 0.42	0.148, 0.44
SBP	0.031, 0.87	0.159, 0.46	0.159, 0.46	0.12, 0.53
DBP	0.259, 0.17	0.207, 0.34	0.059, 0.78	0.098, 0.61
PP	-0.174, 0.37	-0.004, 0.99	0.168, 0.43	0.093, 0.63
IHD	-0.105, 0.58	0.168, 0.44	-0.011, 0.96	0.188, 0.32
ECG abnormality	-0.086, 0.66	-0.054, 0.81	0.036, 0.86	-0.245, 0.19
Drugs (1=yes)				
-ACEi	-0.054, 0.78	0.089, 0.69	-0.058, 0.79	-0.146, 0.44
-ARB	0.262, 0.17	-0.229, 0.29	-0.238, 0.26	-0.347, 0.06
-BBlocker	-0.121, 0.53	0.009, 0.97	0.226, 0.29	0.015, 0.94
-Diuretic (loop)	-0.174, 0.42	-0.573, 0.01	-0.429, 0.07	0.402, 0.05
-Statin	-0.184, 0.34	-0.068, 0.76	-0.035, 0.87	-0.141, 0.46
Log RHI	1	-0.02, 0.92	-0.067, 0.76	-0.122, 0.53
AIx	-0.02, 0.93	1	**0.881, <0.001**	0.049, 0.82
AIx@75bpm	-0.067, 0.76	**0.881, <0.001**	1	0.029, 0.89
Log PWV	-0.122, 0.53	0.049, 0.82	0.029, 0.89	1
EF	0.038, 0.85	0.26, 0.24	0.082, 0.71	-0.249, 0.2
LVMI	-0.118, 0.55	-0.269, 0.23	**-0.55, 0.007**	-0.053, 0.79
cEDV	-0.139, 0.48	-0.162, 0.47	-0.148, 0.44	-0.063, 0.75
cESV	-0.098, 0.62	-0.258, 0.25	0.394, 0.03	-0.394, 0.03
eGFR (6v)	0.119, 0.54	0.096, 0.66	-0.237, 0.27	-0.008, 0.96
Cholesterol	-0.076, 0.69	0.319, 0.14	0.419, 0.04	0.251, 0.18
Cholesterol/HDL	-0.117, 0.55	0.004, 0.99	0.106, 0.62	0.044, 0.82
PTH	-0.171, 0.40	-0.186, 0.42	-0.059, 0.80	-0.308, 0.12
PO4	0.009, 0.96	-0.01, 0.96	0.084, 0.70	-0.073, 0.71
CRP	-0.194, 0.31	-0.103, 0.64	-0.072, 0.74	0.09, 0.64
Hb	-0.071, 0.71	-0.118, 0.59	-0.313, 0.18	0.193, 0.31
25-vit D	-0.283, 0.14	**0.678, <0.001**	0.404, 0.05	0.438, 0.02
Glucose	-0.296, 0.11	-0.282, 0.14	-0.062, 0.74	0.297, 0.11
Urate	-0.197, 0.31	0.016, 0.94	-0.172, 0.37	0.126, 0.52
PCR	-0.17, 0.38	-0.366, 0.09	-0.222, 0.30	0.068, 0.72
Log 24h QP	0.12, 0.55	-0.43, 0.04	-0.162, 0.46	0.316, 0.10
24h USod	-0.154, 0.42	0.033, 0.88	-0.074, 0.73	**0.566, 0.001**
24h USod/U Pot	-0.19, 0.31	-0.17, 0.37	-0.091, 0.63	0.376, 0.04
THALDO	0.164, 0.39	-0.029, 0.88	-0.002, 0.99	0.118, 0.54
THDOC	0.117, 0.54	-0.008, 0.97	0.059, 0.76	0.071, 0.72
Cortisol metabs	0.067, 0.72	0.003, 0.99	-0.259, 0.17	-0.009, 0.96
Log Plasma aldo	-0.405, 0.03	-0.702, 0.71	-0.127, 0.51	-0.126, 0.51
PRC	-0.073, 0.71	0.076, 0.73	0.029, 0.89	-0.25, 0.19
ARR	0.002, 0.99	-0.26, 0.23	-0.213, 0.32	0.23, 0.23

Table 6-3: Correlation matrix in essential hypertensives.
Pearson's or Spearman's correlation coefficient as appropriate. P≤0.01 deemed significant (highlighted in bold).

6.3.3 Reactive hyperaemia index (RHI)

6.3.3.1 RHI in different patient groups

There was no significant difference in RHI between patients with CKD and hypertensives, or between different primary renal diseases (Figure 6-1).

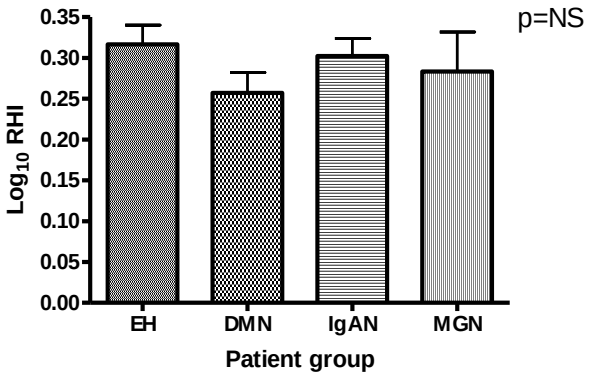


Figure 6-1: Mean (SD) RHI values by patient group.

6.3.3.2 Correlations between demographic factors and RHI

Factors which correlated significantly with a higher RHI in CKD patients (reflecting better endothelial function) (Tables 6-2 and 6-3) were a lower 24h urinary sodium excretion and a higher DBP. Factors approaching significance were lower age (r -0.289, p=0.02), lower PRC (-0.245, p=0.04), lower THALDO (-0.222, p=0.08) and lower THDOC (-0.228, p=0.07) excretion rates.

In EH patients, RHI was lower in females (Table 6-4) and was inversely proportional to PAC.

6.3.3.3 Linear regression of predictors of RHI in patients with CKD

Significant correlates with RHI were entered in a univariate linear regression model to establish significant predictors. Significant univariate predictors of RHI in patients with CKD were DBP and 24h urinary sodium. Both remained significant independent predictors on multivariate analysis. (Table 6-5)

6.3.3.4 Linear regression of predictors of RHI in patients with EH

Female gender (B=0.148 (95% CI 0.029-0.268), β 0.432, p=0.017) and log PAC (B= -0.168 (95% CI -0.315 - -0.021), β -0.405, p=0.026) were significant univariate predictors of log RHI. When both factors were entered into a multivariate linear regression model, neither factor remained significant. PAC was significantly higher in females (p=0.04) with EH than in males.

6.3.3.5 Comparison of CKD patients with RHI > 1.6 compared with <1.6

A cut-off of RHI <1.6 has been previously shown to correlate with increased vascular risk and coronary endothelial dysfunction(255). When this cut-off is applied to patients with CKD the only factor which differs significantly between patient groups is the level of urinary sodium excretion (p=0.002), (Figure 6-2). Therefore, patients with CKD and a higher sodium intake have a greater degree of endothelial dysfunction. Only three patients with EH had a RHI less than 1.6, precluding further analysis.

	EH r	*p*	*CKD R*	*P*
Female gender	0.432	0.02		
Log PAC	-0.405	0.03		
DBP			0.336	0.004
24h USod			-0.349	0.004

Table 6-4: Significant correlates with the reactive hyperaemia index by patient group.

Variable	*Univariate*				*Multivariate*			
	B	*95% CI B*	*β*	*P*	*B*	*95% CI B*	*β*	*p*
DBP(mmHg)	0.004	0.001-0.006	0.334	0.005	0.003	0.001-0.006	0.298	0.013
24h USod (mmol/24h)	-0.001	-0.001-0.000	-0.35	0.004	-0.001	-0.001-0.000	-0.269	0.038

Table 6-5: Significant univariate and multivariate predictors of logRHI in patients with CKD. ***R-squared for multivariate model 0.226.***

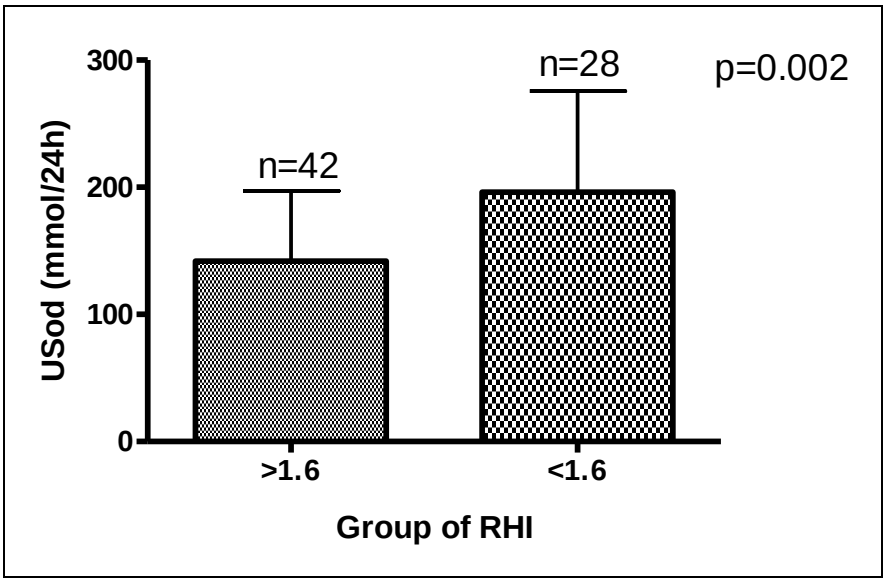


Figure 6-2: Urinary sodium excretion by RHI group.

6.3.3.6 Utility of the reactive hyperaemia index as a measure of endothelial function.

As the reactive hyperaemia index is an index calculated from the degree of increase in signal amplitude in the occluded arm after release of the blood pressure cuff compared with baseline signal amplitude, normalised to the control arm, there is concern that the RHI will be falsely influenced by baseline vasodilatation. In this cohort, the baseline average amplitude on the occluded or control arm did correlate negatively with the RHI i.e. if the average baseline amplitude is high (suggesting vasodilation) the calculated RHI is low (Figure 6-3A). There was however no difference between arms in terms of degree of baseline vasodilatation (Figure 6-3B).

There was no difference between different groups of the cohort in terms of baseline blood flow (one-way ANOVA p=NS) therefore, although the level of baseline vasodilatation is a limitation of the technique it affected all patients similarly.

A possible solution to correct for this limitation, calculating the RHI then multiplying by baseline amplitude in the intervention arm, was carried out but did not alter the study findings.

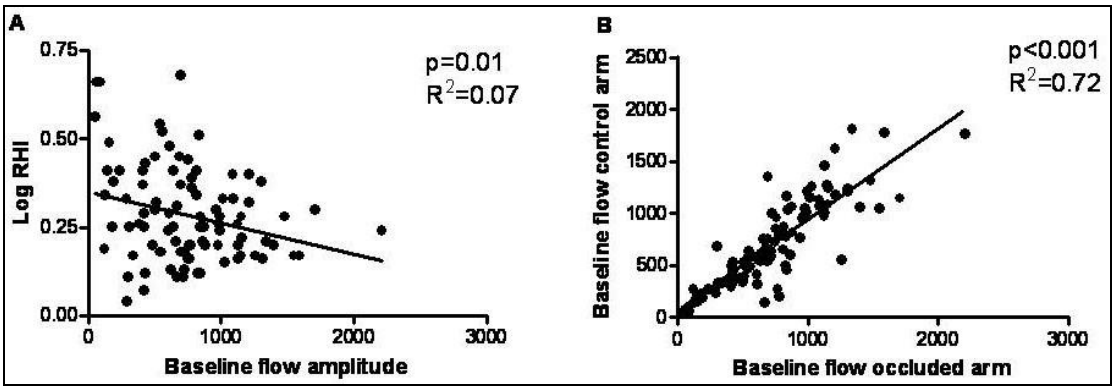


Figure 6-3: A - Scatterplot of baseline flow in the occluded arm versus the derived log reactive hyperaemia index. B - Scatterplot of baseline flow in the occluded arm versus the control arm.

Fitted linear regression line and estimate of significance.

6.3.4 Pulse wave analysis

6.3.4.1 PWA - Comparison between groups.

The augmentation index was significantly higher in patients with EH than in patients with CKD (p=0.02), with the main difference being between patients with EH and patients with IgAN (p=0.03) (Figure 6-4). However, after adjusting for a heart rate of 75bpm, these differences were no longer significant.

6.3.4.2 Correlation between demographic factors and augmentation index in patients with CKD and EH

A correlation matrix was constructed to determine variables which correlated significantly with the AIx@75bpm. There was no significant correlation between AIx@75bpm and level of renal function or different drug treatments (Tables 6-3 and 6-6).

Correlates of AIx@75bpm differed between patients with renal disease and patients with EH (Table 6-6). As expected, in both EH and CKD patients, AIx@75bpm increased with female sex and shorter height.

In CKD patients however there was also a significant correlation between higher AIx@75bpm and lower PRC (p=0.001), lower cortisol metabolites (p=0.002), increased SBP (p=0.001) and PP (p=0.001) and smaller waist circumference (p<0.001). There was also a trend towards lower THALDO excretion and higher AIx@75bpm (p=0.06).

In EH patients, higher AIx@75bpm correlated with a lower LVMI (p=0.007), which may relate to the lower left ventricular mass in women. There was a trend towards higher AIx@75bpm with higher vitamin D levels (p=0.05).

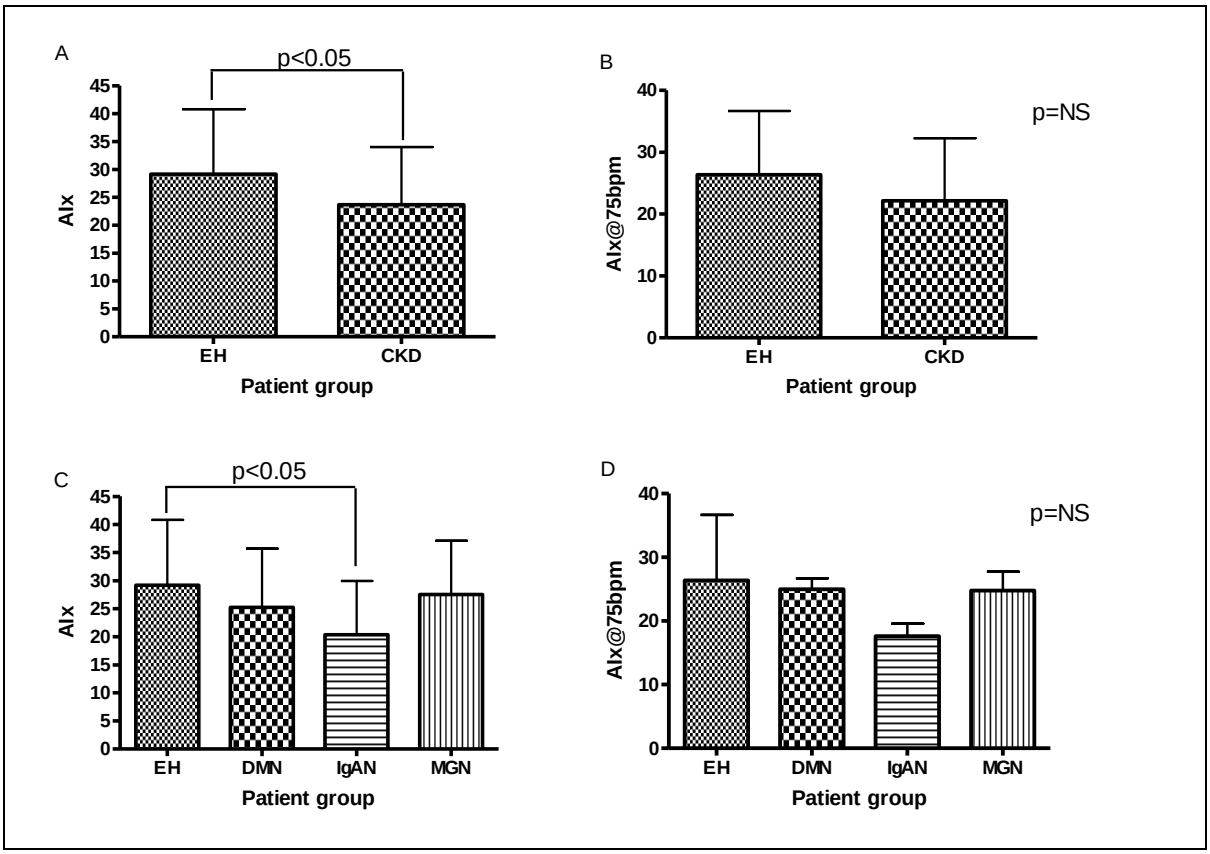


Figure 6-4: Barcharts demonstrating mean (+SD) augmentation index (AIx) and augmentation index @75bpm.

A & B – cohort separated into essential hypertensives and patients with CKD. C & D – cohort separated further by primary renal disease. Comparison by Student's T Test or one-Way ANOVA with Tukey's post-hoc analysis.

	EH		***CKD***	
	r	***p***	***r***	***p***
Female sex	0.624	0.001	0.4	<0.001
Height (cm)	-0.715	<0.001	-0.355	0.003
LVMI (g/m2)	-0.55	0.007		
Weight (Kg)			-0.481	<0.001
Waist circ (cm)			-0.389	<0.001
SBP (mmHg)			0.407	0.001
Pulse pressure (mmHg)			0.411	0.001
Plasma renin concentration (uIU/ml)			-0.389	0.001
Cortisol metabolites (mcg/24h)			-0.388	0.002

Table 6-6: Significant correlations with AIx@75bpm in hypertensive and CKD patients.

P<0.01 = significant.

6.3.4.3 Linear regression of predictors of AIx in patients with CKD

Significant correlates with AIx@75bpm in patients with CKD were entered into a univariate linear regression model to predict AIx@75bpm. Significant univariate predictors of AIx@75bpm in patients with CKD were female gender, height, SBP, pulse pressure, weight, waist circumference, plasma renin concentration and urinary cortisol metabolites (Table 6-7).

Significant univariate predictors were entered together into a multivariate linear regression model. One measure of blood pressure (PP) was entered due to collinearity. Higher pulse pressure, smaller waist circumference and lower PRC remained significant independent predictors of AIx@75bpm.

Variable	*Univariate B*	*95% CI*	*β*	*P*	*Multivariate B*	*95% CI*	*β*	*p*
PP (mmHg)	0.192	0.068-0.316	0.36	0.003	0.158	0.04-0.27	0.30	0.009
Waist circ (cm)	-0.333	-0.506- -0.106	-0.445	<0.001	-0.298	-0.46- -0.14	-0.40	<0.001
√ PRC	-0.548	-0.904- -0.192	-0.359	0.003	-0.37	-0.703- -0.04	-0.24	0.03
Female gender	7.33	1.76-12.9	0.312	0.011				
Height (cm)	-0.34	-0.576- -0.104	-0.339	0.005	-	-	-	-
SBP (mmHg)	0.152	0.048-0.255	0.345	0.005	-	-	-	-
Weight (Kg)	-0.246	-0.359- -0.134	-0.479	<0.001	-	-	-	-
Cortisol metabolites (mcg/24h)	-0.001	-0.001-0.000	-0.388	0.002	-	-	-	-

Table 6-7: Univariate and multivariate linear regression models of predictors of AIx@75bpm in patients with CKD.
R-squared for multivariate model = 0.49.

6.3.4.4 Multivariate analysis of the significant predictors of AIx in essential hypertensives

Gender, height and logLVMI correlated significantly with AIx@75bpm in patients with EH and were entered into a univariate linear regression model, whereby only gender and height remained significant univariate predictors. On multivariate analysis only height (cm) remained a significant independent predictor of AIx@75bpm (B=-0.651, 95% CI -1.17- -0.14, p=0.016) in patients with EH.

In patients with EH, AIx@75bpm is a univariate predictor of LVMI on linear regression – the lower the AIx@75bpm, the higher the LVMI (B= -0.005 (95% CI -0.008 - -0.001), p=0.015). However, when gender is added to the model, neither is a significant independent predictor.

6.3.5 Carotid-femoral pulse wave velocity

6.3.5.1 Levels and correlations with patient demographics

PWV was significantly higher in patients with CKD compared with EH (p=0.006), with highest levels in patients with diabetic nephropathy (p<0.001) (Figure 6-5), although they had lower levels of renal function. In all patients PWV was associated with eGFR (figure 6-6).

Significant correlations between PWV and demographic factors differed between patients with CKD and patients with EH (Table 6-9). In CKD patients, higher PWV correlated significantly with older age, higher SBP, higher pulse pressure, lower eGFR, higher serum glucose, higher 24h USod:UPot ratio, larger waist circumference, lower haemoglobin and also correlated with the presence of ECG abnormalities. There was a trend towards higher PWV and higher LVMI (p=0.05), lower serum cholesterol (p=0.02), higher PTH (0.05), higher serum phosphate (0.02) and higher 24h urinary sodium excretion (0.04).

In EH patients, a higher PWV correlated significantly with higher urinary sodium excretion. There was also a trend towards higher PWV with higher vitamin D levels (p=0.02) in these patients.

There was a significant relationship on linear regression analysis between number of antihypertensive medications and PWV (p=0.014).

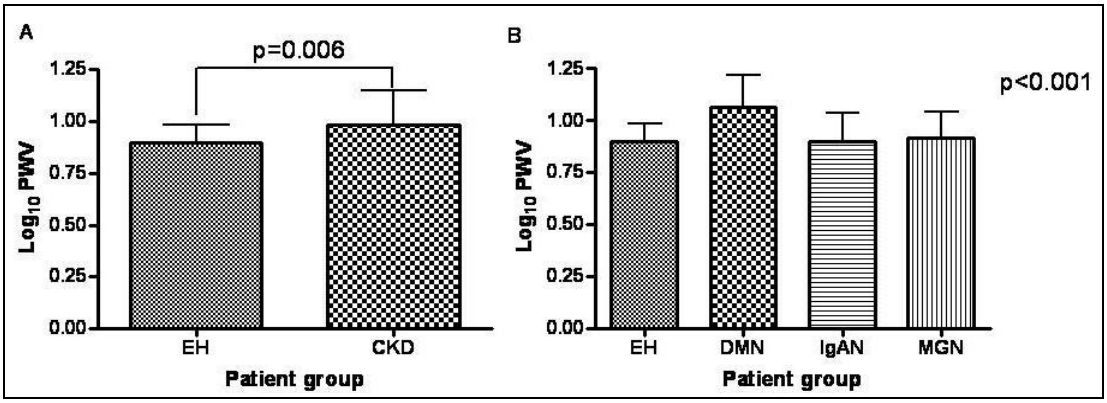


Figure 6-5: Bar chart of PWV by patient group.
Data expressed as mean (SD). Comparison by Student's T-test (A) or one-way ANOVA (B).

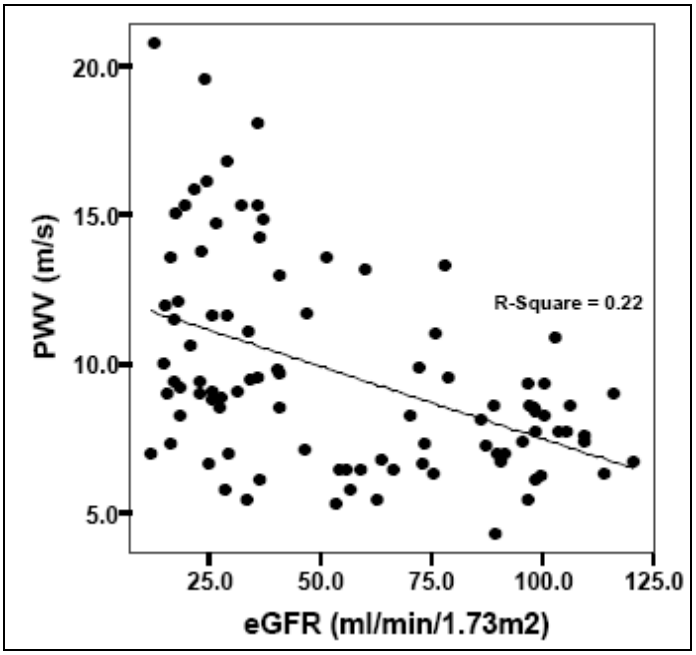


Figure 6-6: Scatterplot with fitted linear regression line demonstrating association between eGFR and PWV.

6.3.5.2 Linear regression of predictors of PWV in patients with CKD

Factors which correlated significantly with PWV in patients with CKD were entered into a univariate linear regression model. From this, significant univariate predictors of PWV were age, waist circumference, SBP, pulse pressure and eGFR. On multivariate analysis, when all significant univariate predictors were entered into a multivariate linear regression model (including only one measure of blood pressure, PP), only pulse pressure, waist circumference and age remained significant independent predictors (Table 6-9).

	EH r	*p*	*CKD r*	*P*
24h USod	0.566	0.001		
Age			0.602	<0.001
Waist circumference			0.286	0.008
SBP			0.373	0.002
Pulse pressure			0.516	<0.001
eGFR			-0.468	<0.001
24h USod/UPot			0.37	0.003
ECG abnormality			0.286	0.007
Serum glucose			0.347	0.006
Haemoglobin			-0.337	0.007

Table 6-8: Correlations with logPWV reported by patient group.

Variable	*Univariate*				*Multivariate*			
	B	*95% CI*	*β*	*P*	*B*	*95% CI*	*β*	*P*
Age (y)	0.008	0.005-0.010	0.602	<0.001	0.005	0.003-0.008	0.432	<0.001
Waist circ (cm)	0.004	0.001-0.007	0.349	0.006	0.003	0.001-0.005	0.241	0.015
Pulse pressure (mmHg)	0.004	0.002-0.006	0.516	<0.001	0.002	0.000-0.004	2.55	0.012
Systolic BP (mmHg)	0.002	0.001-0.004	0.373	0.002				
eGFR (ml/min/1.73m^2)	-0.003	-0.005-0.002	-0.468	<0.001				
Haemoglobin (g/dl)	-0.031	-0.053-0.009	-0.337	0.007				

Table 6-9: Significant univariate and multivariate predictors of logPWV in patients with CKD.
Only pulse pressure, not SBP, was entered into the multivariate model due to its higher predictive value and collinearity between the two variables. R-square value for the multivariate model was 0.59.

6.3.5.3 Linear regression of predictors of logPWV in patients with EH

24h urinary sodium excretion was the only significant correlate with PWV in patients with EH. On linear regression analysis it remained a significant predictor (B=0.001, (95% CI 0.0-0.001), p=0.001, R2=0.32).

6.3.6 Endothelial function, pulse wave velocity and augmentation index - association with proteinuria and left ventricular abnormalities

RHI, PWV and AIx@75bpm measures did not correlate with levels of proteinuria, ventricular mass, left ventricular ejection fraction or left ventricular volume in patients with CKD. PWV correlated significantly with the presence of ECG abnormalities in patients with CKD. Using binary logistic regression with ECG abnormalities as the predicted variable, logPWV was a significant univariate predictor (B=4.57, p=0.016).

In patients with EH, AIx@75bpm correlated significantly with LVMI, however was not a significant multivariate predictor. RHI, PWV and AIx measures did not correlate with left ventricular ejection fraction or left ventricular volume.

6.4 Discussion

6.4.1 Key findings

In this study it has been demonstrated that :

- The determinants of markers of vascular function differ between patients with EH and CKD.
- Urinary steroid excretion was not associated with vascular function measures.
- Urinary sodium excretion was the only significant determinant of PWV in patients with EH and an independent predictor of RHI in patients with CKD.
- Plasma aldosterone concentration was a univariate predictor of RHI in patients with EH, but not a multivariate predictor.

- None of the three vascular or endothelial function methods tested were independently associated with left ventricular abnormalities or proteinuria excretion in patients with CKD.

6.4.2 Carotid-femoral PWV

Carotid-femoral pulse wave velocity is the gold standard measure of vascular stiffness. In this CKD cohort, the significant independent predictors of PWV were :

- Increasing age
- Increasing waist circumference
- Higher pulse pressure

Although PWV was higher in patients with reduced renal function, this was not an independent association once adjustments were made for other demographic factors. There was no significant association between THALDO, THDOC or urinary sodium excretion and PWV. In patients with CKD, PWV predicted the presence of ECG abnormalities but was not associated with cardiac MRI determined LV mass, function or volume. Nor was it associated with the level of proteinuria excretion.

In patients with EH, PWV was independently determined by 24h urinary sodium excretion, a reflection of dietary sodium intake.

6.4.3 Augmentation index

Augmentation index is an integrated measure of vascular stiffness, with a higher value being an indicator of poor prognosis. In both groups AIx@75bpm increased with increasing height and was higher in females, which are recognised associations.

In CKD patients however, the following were significant independent predictors, removing the association with height and gender :

- Increasing pulse pressure

- Smaller waist circumference

- Lower plasma renin concentration

It may be in renal patients that intravascular volume expansion suppresses PRC and raises pulse pressure, resulting in a higher AIx.

In EH patients, AIx@75bpm was independently predicted by height, with the time taken for the reflective wave to return being longer with the further distance to travel. AIx@75bpm was a significant negative predictor of LVMI in these patients, although this was likely due to AIx being higher in women, who have a lower LVMI.

6.4.4 Reactive hyperaemia index

The reactive hyperaemia index is a nitric oxide mediated measure of endothelial function. The RHI falls as the endothelium becomes dysfunctional. In patients with CKD, high urinary sodium excretion was independently associated with a lower RHI. Diastolic blood pressure was also a significant multivariate predictor of the RHI – the higher the DBP, the higher the RHI, which is contrary to what one would expect, but may be explained by peripheral vasoconstriction, increasing DBP and RHI.

In patients with EH, univariate (but not multivariate) predictors of low RHI were :

- Male gender

- Increasing PAC

Measure	CKD	EH
PWV	↑ Age ↑ Waist circ ↑ Pulse pressure	↑ 24h USod
AIx@75bpm	↑ Pulse pressure ↓ Waist circ ↓ PRC	↑ Height
RHI	↑ 24h USod ↓ DBP	

Table 6-10: Significant multivariate independent predictors of measures of vascular function in patients with CKD and patients with EH.

6.4.5 Comparison with previous studies

In normal subjects, PWV increases with age and blood pressure(314) and is higher in patients with diabetes(315). Increasing PWV with increasing waist circumference has also previously been reported in normal individuals(316). Similarly, augmentation index relates to diastolic blood pressure, height and gender(317) – being higher in women for reasons that are unclear(318). Endothelial function, measured as the RHI, has been shown to be independently associated with male gender, BMI, cholesterol, diabetes, age and smoking(256).

The most comprehensive CKD cohort with which to compare our findings is from America. Townsend et al assessed determinants of aortic PWV using Sphygmocor carotid-femoral PWV in a CRIC substudy(319), utilising measurements in 2564 patients with CKD. Similar to our findings, independent predictors of PWV were increasing age, blood pressure and waist circumference, as well as serum glucose, black race and male gender. They found each 10ml/min/1.73m^2 loss in eGFR to be associated with a 0.4m/s increase in PWV, although eGFR was not a significant independent predictor. There was also no significant independent relationship between PWV and proteinuria.

Covic et al(238) assessed independent predictors of arterial stiffness parameters in patients with CKD. Similar to our cohort, age predicted PWV and MAP independently predicted AIx. Toussaint et al(320) reported an association between PWV and age in patients with CKD, but also found triglyceride levels to be significant independent predictors. We found no association with lipids, but did not assess triglycerides directly.

Using the radial augmentation index in non-diabetic patients with CKD 3, Takenaka et al(244) found the independent predictors of AIx to be increasing age and weight, shorter height and lower heart rate. These findings are contrary to our own, perhaps reflecting a differing case mix. They also found increasing AIx was associated with LVMI (on echo) in male patients, which we did not; and showed AIx to be a significant independent predictor of slope decline in eGFR.

There are no published data assessing RHI in patients with CKD with which to compare our findings, however we were unable to demonstrate the independent associations seen in the Framingham cohort (male sex, BMI, waist circumference, SBP, DBP and lipids). The RHI is an attractive operator independent method of measuring NO dependent endothelial function. It is however limited by baseline vasodilation. Alternative ways of measuring endothelial function such as the gold standard brachial flow mediated dilatation (FMD) have not been directly compared with EndoPAT in patients with CKD, to confirm that it is a valid measurement.

We found 24h USod excretion to be an independent predictor of PWV in patients with EH. In a heterogenous normotensive and hypertensive Portugese population, carotid-femoral PWV was independently determined by age, SBP and urinary sodium excretion(321). In a black South African cohort, urinary sodium alone was not associated with PWV or AIx, but when corrected for urinary potassium excretion, it was an independent predictor of AIx(322).

The finding that urinary sodium excretion was a strong predictor of RHI in patients with CKD is novel. The findings support the notion that elevated sodium intake contributes to endothelial and vascular dysfunction, potentially leading to increased risk of mortality. The mechanisms by which sodium has a detrimental effect are not clear but are likely to include oxidative stress or volume overload.

An Australian cross-over study in normotensive individuals demonstrated that a low salt diet can significantly improve endothelial function (measured using FMD) independent of blood pressure, but had no effect on augmentation index or pulse wave velocity (323). The trial lasted only 2 weeks and it may be that to have an effect on larger arteries, a longer trial period would be required.

The finding that urinary steroid excretion and PAC are not related to measures of vascular function is surprising. The MCR is known to be expressed on vascular endothelium and indeed local systems are thought to exist which mediate vasoconstriction. Similarly, it is unclear why treatment with spironolactone should reduce PWV in patients with CKD(131), when urinary steroid excretion was not associated with the measure. Perhaps local RAAS systems are not reflected in serum or urine measurements of steroids.
Alternatively, reduction in blood pressure is the dominant mechanism of benefit.

6.4.6 Limitations

The number of patients assessed in the EH cohort was small, meaning that weaker correlations would not be identified. Small sub-groups within the CKD cohort mean further analysis was limited. All patients included in the study were prescribed anti-hypertensive medications, including ACEi, which modify PWV(324) and endothelial function and beta blockers which reduce augmentation index, meaning that some associations may be masked. As a cross-sectional analysis, implications regarding the utility of these measures in prognosis cannot be made. Similarly, causation and directionality cannot be implied by the reported associations.

6.5 Conclusions

The determinants of vascular and endothelial function in patients with EH and CKD differ. We have confirmed previously reported associations between measures. The role of renal function in determining the measures of vascular function appears to be indirect and explained by other factors such as obesity and blood pressure, as eGFR was not an independent predictor on multivariate analysis. Urinary sodium excretion is an independent predictor of endothelial dysfunction but urinary steroid excretion was not associated with any of the three measures.

7 Chapter Seven: An in vitro study of the effects of aldosterone and sodium on proximal tubular cells

7.1 Introduction

In addition to its traditional physiological role of sodium retention, aldosterone has also been shown to have pro-inflammatory and pro-fibrotic effects in rats, in the context of a high sodium environment, leading to glomerulosclerosis and interstitial fibrosis (141;146).

Fibrosis involves the accumulation of extracellular matrix, usually collagen, in response to injury or inflammation. In renal disease, tubulointerstitial fibrosis has consistently been shown to be the predominant histological marker of the risk of renal progression(25). Renal fibrosis is mediated via mesenchymal cells (the interstitial fibroblast or glomerular mesangial cell), cells which migrate from the vasculature or via epithelial-mesenchymal transition (325).

Proximal tubular cells are also thought to be important in the development of interstitial fibrosis. HK-2 cells are an immortalized human proximal tubular cell line, known to express the MR (187), however the pro-inflammatory and pro-fibrotic effects of Aldo on proximal tubular cells, a non-traditional mineralocorticoid target, have not been determined. Similarly, despite the in vivo studies where a high sodium environment is necessary for pathological effects, the combined effects of Aldo and sodium on renal cells have also not been studied. Evidence from cardiomyocytes suggests Aldo inhibits the hyperosmolar cell shrinkage of a high sodium environment, leading to cell hypertrophy(177).

The aims of this study were therefore

1. To confirm that HK-2 cells respond to Aldo.

2. To ascertain whether HK-2 cells produce pro-inflammatory cytokines in response to Aldo, and whether this is modified by a high sodium environment.

3. To establish whether there is evidence of a pro-fibrotic response to Aldo by HK-2 cells in terms of collagen production, and whether this effect is a generic MR effect or ligand specific.

7.2 Methods

The laboratory techniques, chemicals and immunobiological agents used in this chapter are described in detail in chapter 2.

7.2.1 Statistical analysis

Differences between groups were assessed using one-way analysis of variance or Student's t-test as appropriate. Analyses were conducted using SPSS (Illinois) v.15.0.

7.3 Results

7.3.1 HK-2 cells express the mineralocorticoid receptor

To confirm that HK-2 cells express the MR reverse transcription PCR was performed using cDNA synthesised from untreated HK-2 cells as described in the Methods chapter. The PCR product was then run on an agarose gel and analysed qualitatively under UV light. This confirmed that HK-2 cells express the MR by the presence of an appropriately sized band (Figure 7-1).

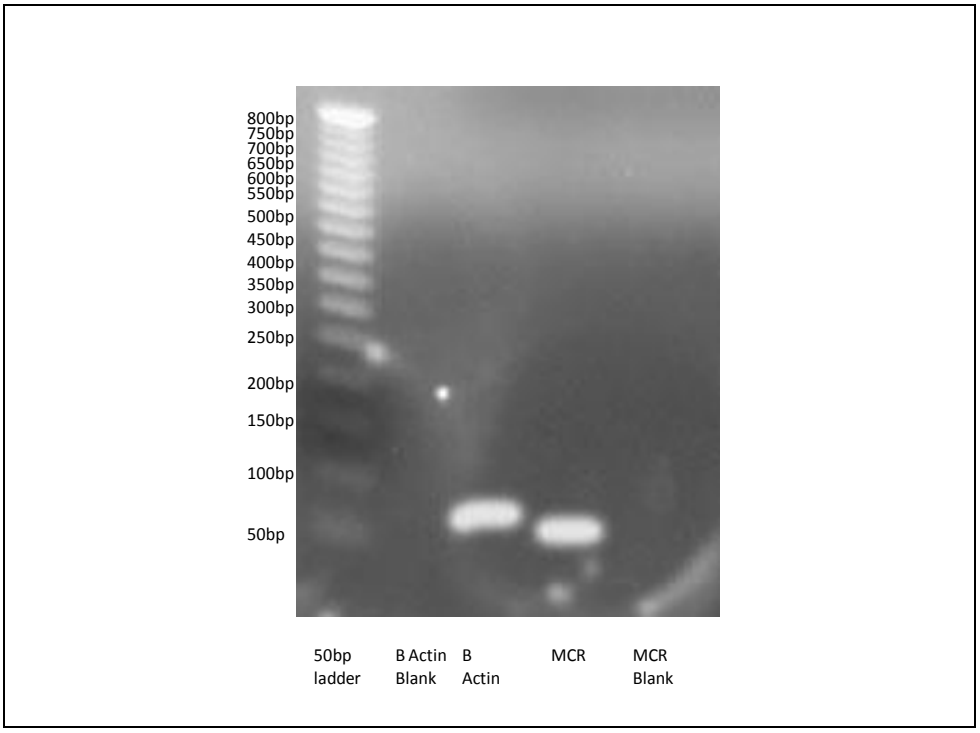


Figure 7-1: Ethidium bromide stained agarose gel viewed under UV light.

Amplification of the MCR cDNA fragment (amplicon size 73bp) and Beta actin amplicon 97bp.

7.3.2 HK-2 cells phosphorylate ERK in response to Aldo treatment

In order to confirm that HK-2 cells were responsive to Aldo treatment (see section 1.4.4, non-genomic signalling) an ERK phosphorylation assay was performed. HK-2 cells were serum starved overnight and treated with Aldo (10^{-6}M) for 1, 5, 10 and 15 minutes. One control well was stimulated with fetal bovine serum (FBS) for 15 minutes. Western blot was performed in triplicate which confirmed that HK-2 cells responded in a time-dependent manner to Aldo stimulation by phosphorylating ERK, maximally at 5 minutes. A representative plot is shown (figure 7-2).

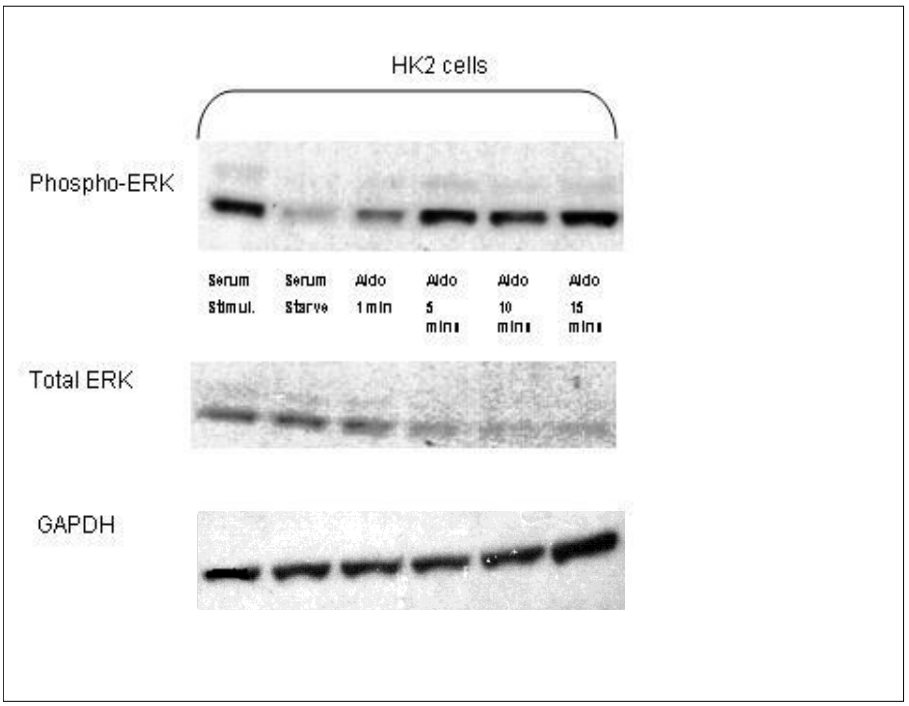


Figure 7-2: Representative immunoblot demonstrating time dependent ERK phosphorylation after treatment with FBS (serum stimulation).

Aldo (10^{-6}M) treatment for 1 minute, 5minutes, 10 minutes and 15 minutes. GAPDH was used to demonstrate equal loading.

7.3.3 Aldosterone or angiotensin II treatment alone or in combination with sodium does not result in an increase in IL-6, IL-8, GM-CSF or TGF-β1 production measured using ELISA

To ascertain whether treatment of HK-2 cells with Aldo and or sodium resulted in a pro-inflammatory response, ELISA was performed on cell culture supernatant. HK-2 cells were seeded at a density of $2x10^5$ and incubated for 1, 24 or 48 hours in predetermined culture conditions. The 1h time point was chosen to ascertain whether any detected effect was genomic or non-genomic (see section 1.4.4).

Cells were cultured with sodium alone (Na) (additional 50mmol/l - total media concentration 150mmol/l), Aldo alone at differing concentrations (Aldo 10^{-6} M or Aldo 10^{-8} M) or both. The concentration of sodium was chosen as it was thought that the sodium content of the proximal tubule would be higher than normal serum concentration (135-145mmol/l). The Aldo concentration was chosen to reflect physiological levels (10^{-8}) and supraphysiological levels. Commencing Aldo treatment at a 1nM concentration is reasonable as this is consistent with the reported Kd for Aldo-MR binding (326).

Control cells were stimulated with lipopolysaccharide (LPS). ELISA was then performed on cell culture supernatant and results were normalised to untreated cells (negative control). Experiments were repeated 6 times. There was no significant difference in IL-6, IL-8 or GM-CSF production from baseline in response to Aldo or sodium at 1hour, 24 hours or 48 hours (Figure 7-3). GM-CSF was not detectable at 1h. HK-2 cells did not excrete TGF-β1 at 24 or 48 hours either basally or in response to LPS, Aldo or sodium at the above concentrations and is therefore not included in the figure.

Cells were also treated with angiotensin II at a concentration of 10^{-6}M alone and in combination with Aldo 10^{-8} M and sodium to assess whether there was a synergistic or permissive effect of AgII. No response was seen in terms of IL-6, IL-8 or GM-CSF production at 48 hours (Figure 7-4).

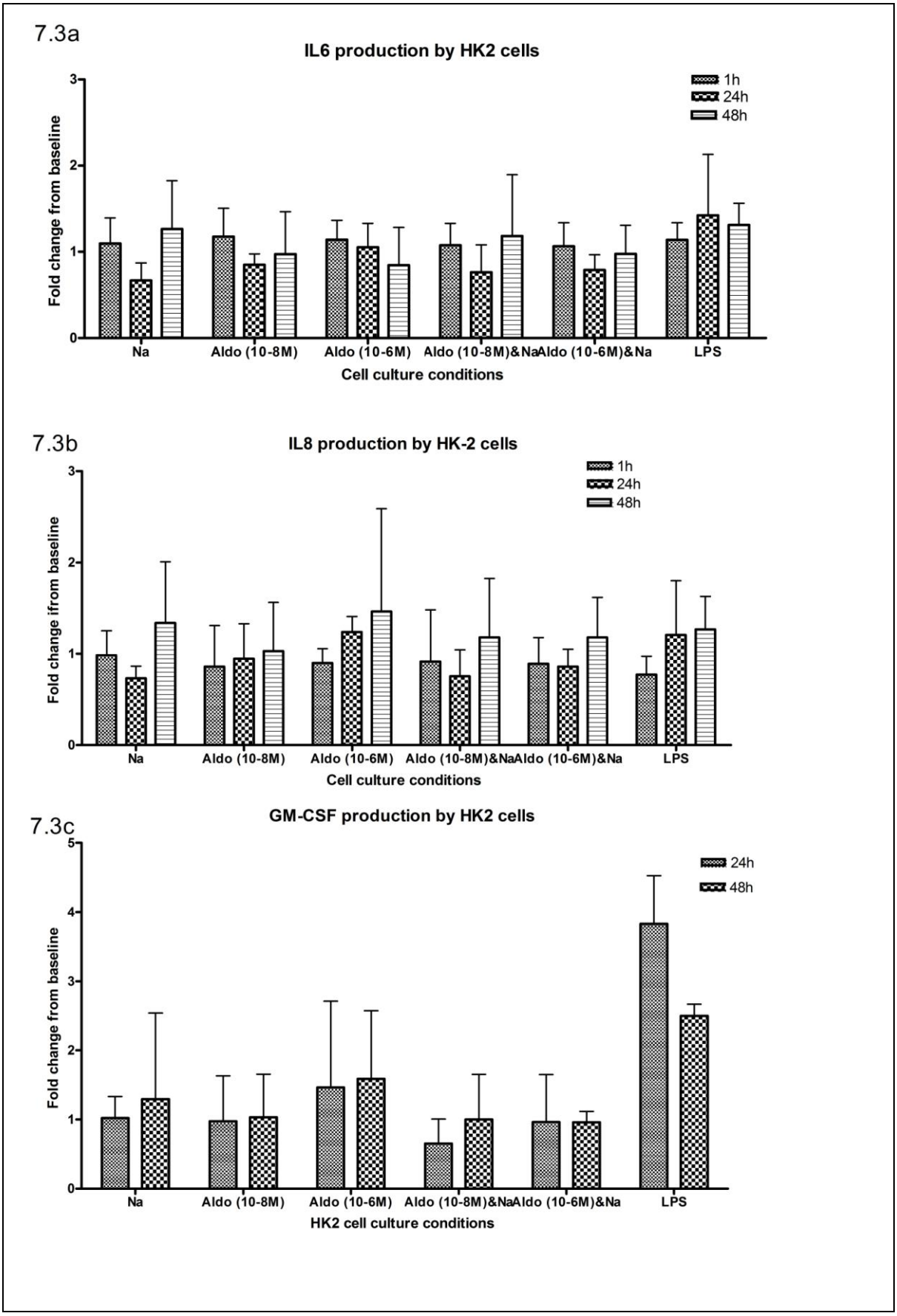


Figure 7-3: Mean fold change from baseline in cytokine production by HK-2 cells measured using ELISA.

7.3a: IL6. 7.3b: IL8. 7.3c: GM-CSF. Results normalised to negative control. See text for definitions of cell culture conditions. Comparisons made using one-way ANOVA, not including LPS stimulation.

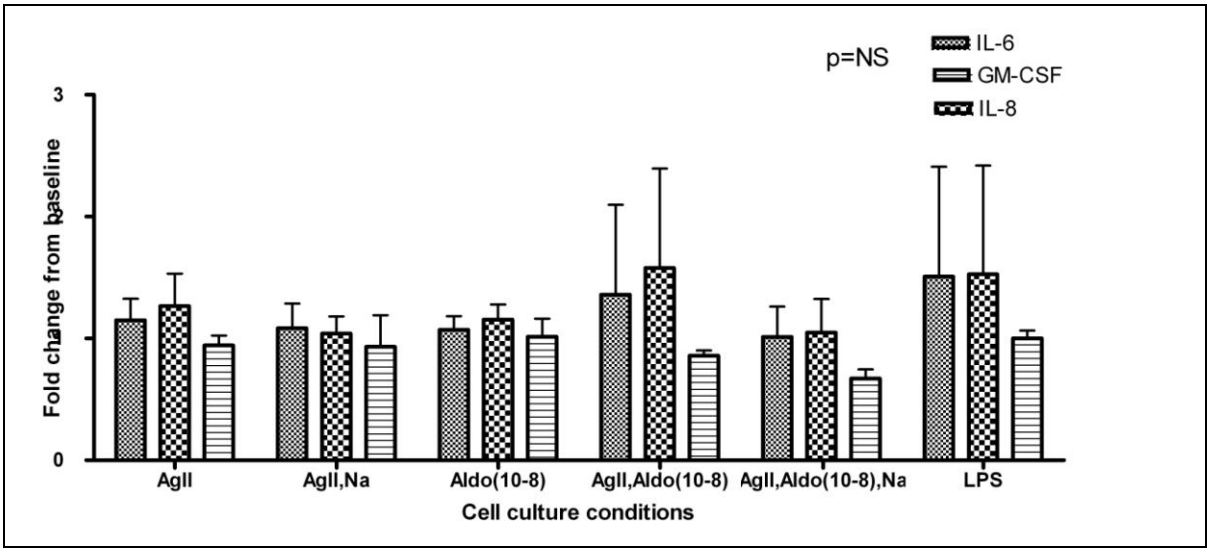


Figure 7-4: Mean fold change in cytokine production (detected by ELISA) by HK-2 cells after angiotensin II treatment.

7.3.4 Transforming growth factor beta gene expression is not influenced by aldosterone or sodium treatment.

As the dominant inflammatory cytokine associated with interstitial fibrosis(160), this mediator was explored further, as cell culture studies suggested it was not released by HK-2 cells. To ascertain whether TGF-β1 was being synthesised but not secreted, or whether TGF- β2 or β3 were being synthesised rather than TGF- β1, real time PCR was undertaken using HK-2 cells treated with Aldo (Aldo 10^{-6} M or Aldo 10^{-8} M) alone or in combination with sodium (additional 50mmol/l, total concentration 150mmol/l) for 24 and 48 hours. Beta actin was amplified as the house-keeping gene to which results were normalised and each experiment was replicated six times. There was no significant change in TGF-β1, 2 or 3 gene expression with experimental conditions (Figure 7-5).

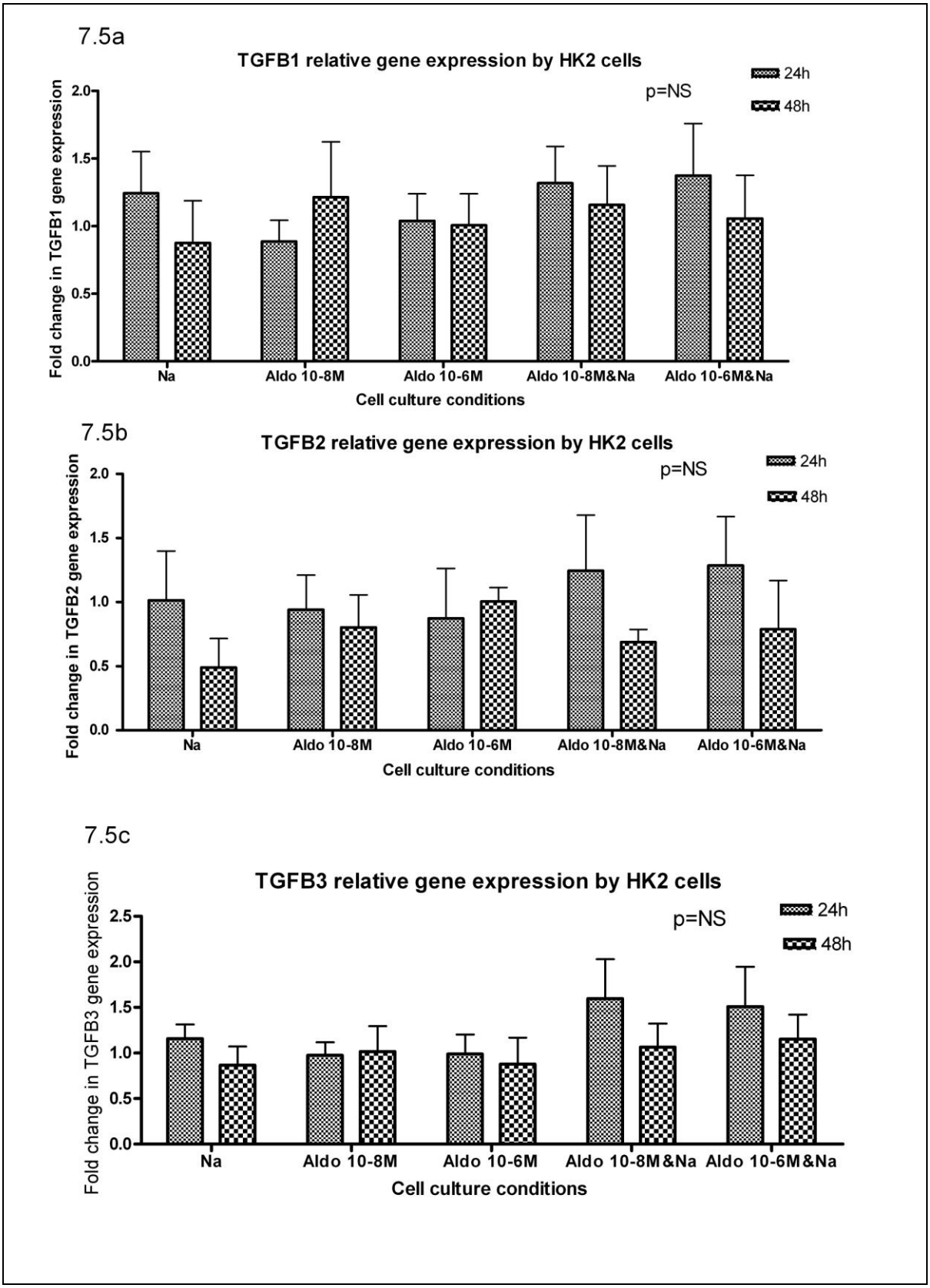


Figure 7-5: Mean fold change in TGFβ gene expression by HK-2 cells relative to baseline in response to sodium and aldosterone treatment.

a: TGF β1; b: TGF β2, c: TGF β3. Results compared by 0ne-way ANOVA for 24h and 48h treatment.

7.3.5 Collagen 1 gene expression is up-regulated in a high sodium and aldosterone / cortisol environment – an effect which is blocked with canrenone.

To ascertain whether HK-2 cells mounted a pro-fibrotic response to treatment with Aldo and sodium, real time PCR was undertaken for collagen 1 and collagen 4 (C1 and C4) gene expression. HK-2 cells were treated with Aldo (Aldo 10^{-6} M or Aldo 10^{-8} M) alone or in combination with sodium (additional 50mmol/l, total concentration 150mmol/l) for 24 and 48 hours. C1 and C4 were chosen because these are the primary constituents of renal interstitial fibrosis.

There was no significant difference in C4 relative gene expression under differing experimental conditions (Figure 7-6).

Aldo or sodium alone did not result in a significant increase in C1 gene expression at 24h or 48h. However, at 48h, compared with Aldo10^{-6} alone ($p=0.002$) or Na alone ($p=0.007$), Aldo10^{-6} and Na together significantly increased gene expression 2.2 fold (SD1.03). Aldo10^{-8} and Na resulted in a 2.54 fold (SD 0.83) increase, which was significantly higher than Aldo10^{-8} alone ($p=0.016$) and Na alone ($p=0.009$) at 48h (Figure 7-7).

Similar results were seen at 24h, with Aldo10^{-6} and Na significantly increasing collagen 1 gene expression 2.85 fold (SD 1.4); and Aldo10^{-8} and Na significantly increasing collagen 1 gene expression 1.79 fold (SD 0.31) ($p<0.05$ for all comparisons) (Figure 7-7).

The addition of canrenone significantly abrogated this synergistic response at 24h and 48h with both Aldo10^{-8} and Na and Aldo10^{-6} and Na (Figure 7-7).

To ascertain whether this was a specific Aldo effect or due to ligand binding to the MR, HK-2 cells were incubated for 48h with cortisol 10^{-9}M alone (dose utilised in previous studies (152)) and in combination with sodium (media concentration 150mmol/l). Again, cortisol alone did not result in a relative increase in C1 gene expression but the combination of cortisol and sodium increased relative C1 gene expression 3.02 fold (SD 0.39). This effect was significantly inhibited by the addition of canrenone (10^{-6}M) ($p=0.005$), but not back to baseline (Figure 7-8). Co-culture of cells with lower dose cortisol (10^{-11}M) and sodium resulted in a similar increase in relative C1 gene expression 2.65 fold (SD 0.16).

Therefore, collagen 1 gene upregulation is a generic effect seen secondary to binding of the MR in HK-2 cells and is not a ligand specific effect.

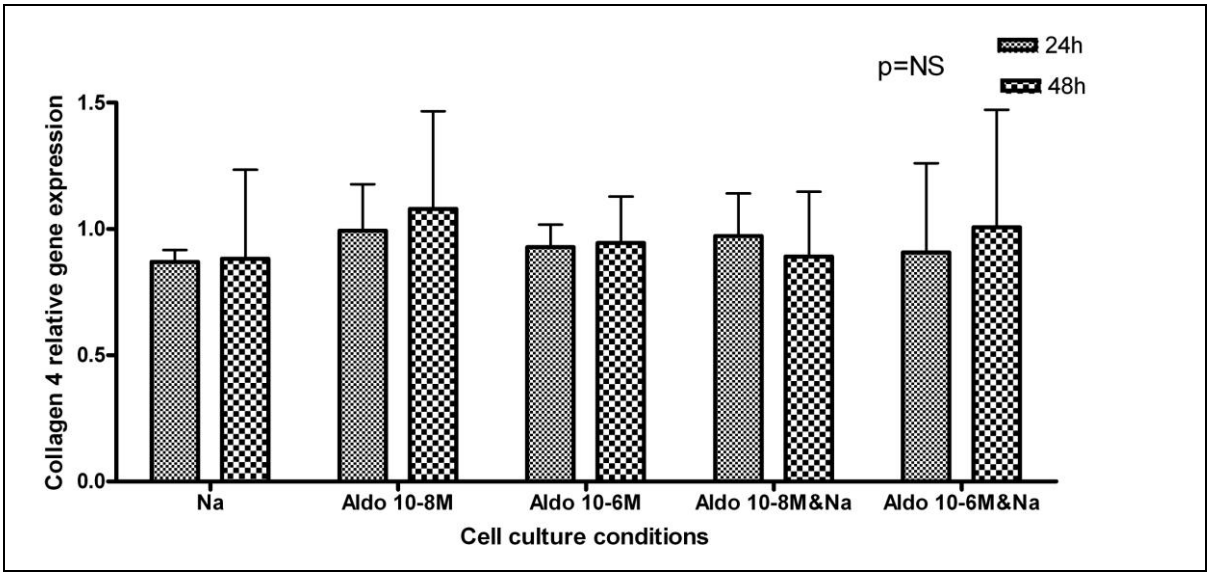


Figure 7-6: Fold change in collagen 4 production from baseline at 24h and 48h in response to treatment with aldosterone and sodium.

Mean (SD). Comparison by one-way ANOVA.

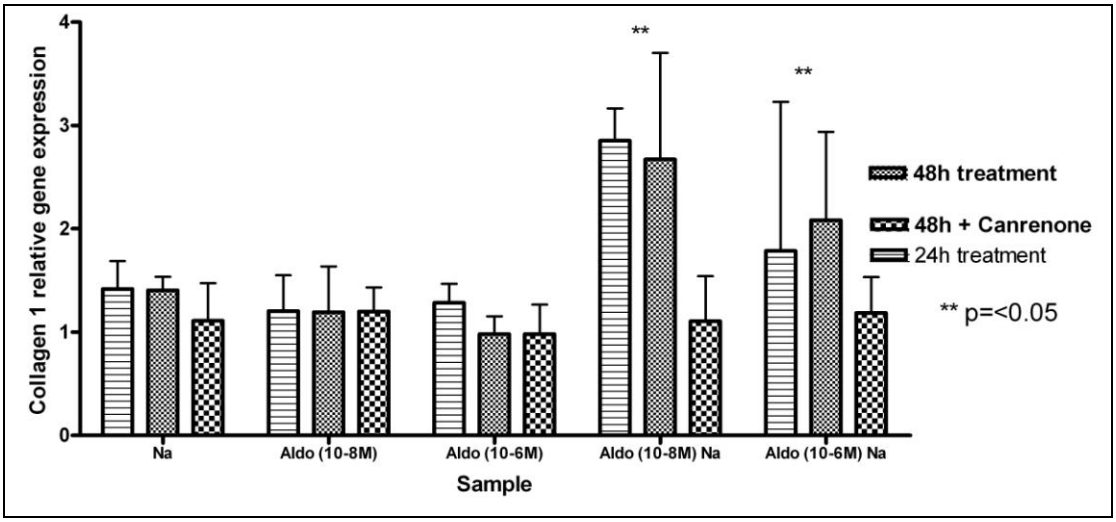


Figure 7-7: Change in collagen 1 gene expression in HK-2 cells treated with Aldo, sodium and canrenone for 24h and 48h.
Mean (SD). Comparison by one-way ANOVA.

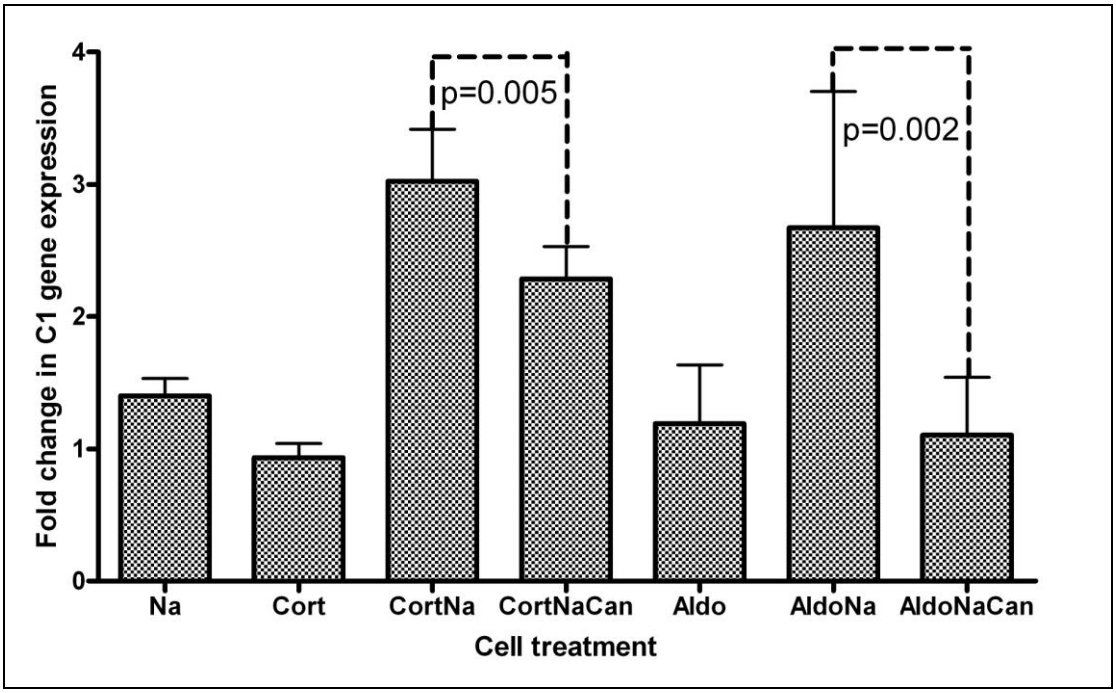


Figure 7-8: Relative change in collagen 1 gene expression after 48h treatment of HK-2 cells. ***Comparison by t-test. Data expressed as mean (SD).***

7.3.6 11-beta hydroxysteroid dehydrogenase type 2 is expressed by HK-2 cells

To ascertain whether the effect of cortisol seen at the MCR in HK-2 cells was secondary to an absence of the 11βHSD2 enzyme, which usually protects the MCR from non-Aldo steroid binding, reverse transcription was performed and PCR was undertaken for the 11βHSD2 enzyme and the product run on an ethidium bromide stained gel. An appropriately sized band was seen under UV light, demonstrating that HK-2 cells do express the 11βHSD2 enzyme gene (Figure 7.9).

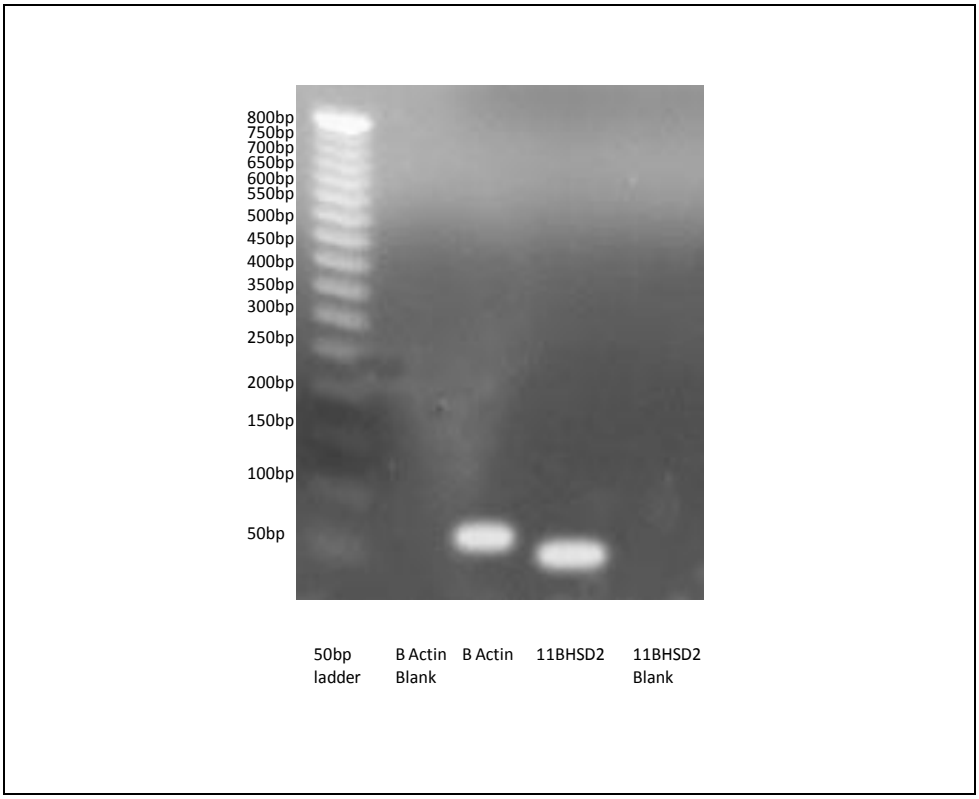


Figure 7-9: HK-2 cells express 11BHSD2.

Unstimulated HK-2 cell cDNA amplified using primers specific for the 11βHSD2 enzyme and run on an ethidium bromide gel. 11βHSD2 enzyme amplicon size 65bp. BActin control amplicon 97bp.

7.3.7 Western blot analysis of collagen 1 expression

Attempts were made to quantify collagen 1 protein expression using Western blot analysis. Two different antibodies were tried under differing assay conditions but the assay could not be accurately reproduced.

7.4 Discussion

It can be seen that HK-2 cells, traditionally viewed as a proximal tubular cell line, have a mixed phenotype and express the MR, allowing them to respond to Aldo, as evidenced by time-dependent ERK phosphorylation. They also express the enzyme 11-beta hydroxysteroid dehydrogenase type 2 (11-BHSD2). This mixture of phenotype enables the study of the effects of Aldo treatment and MR activation on renal cells.

The following are the key findings of this study on the effects of aldosterone and sodium on human proximal tubular cells:

1. Collagen 1 gene expression increases in response to treatment with Aldo or cortisol at physiological concentrations, but only in a high sodium environment.

2. Blockade of these effects using canrenone, an MR antagonist, confirms that this is a MR-mediated effect. Collagen 1 is the main component of renal interstitial fibrosis and this novel finding provides further evidence for the synergistic role of aldosterone and sodium in the development of fibrosis.

That Aldo has renal effects beyond its traditional distal tubular role is further supported in the literature by the localisation of the MR to sites out with the distal nephron, including mesangial cells(327) and podocytes(193). It has also been localised to the (renal) vascular endothelium(328) and vascular smooth muscle cells(329), where it is protected by the enzyme 11BHSD2.

The findings in this study support in vivo evidence that mineralocorticoids have a direct pro-fibrogenic effect on the rat kidneys and heart, in the context of a high sodium environment. These studies are described in section 1.7. The convincing in vivo findings have led to more focused in vitro attempts to clarify the exact molecular in intracellular pathways involved in this pro-inflammatory pro-fibrotic response.

The effects of Aldo have been studied in a number of different renal cell types including fibroblasts, mesangial cells, human kidney cortex cells and inner medullary collecting duct cells, with evidence for an increase in inflammatory mediators and collagen production in response. These studies are described in section 1.8.7.

There are however no studies demonstrating a direct pro-inflammatory effect of Aldo on HK-2 cells. In the studies reported here, there was no evidence that Aldo directly influences IL6, IL8, TGFβ1 or GM-CSF production. It may be that effects are mediated via other cytokines which were not tested e.g. IL-1 or PAI-1, or it may be that additional cells are required for Aldo to have pro-inflammatory effects e.g. fibroblasts in the interstitium. Similarly, sodium alone or in combination with Aldo had no direct effect on a pro-inflammatory phenotype.

Therefore there is a large body of evidence suggesting a direct pathological role for Aldo in the kidney. These effects appear to be mediated via the MR, with subsequent ERK phosphorylation and intracellular signalling. This study has confirmed that activation of the MR in cells of renal origin, in a high sodium environment, can result in a pro-fibrotic phenotype. Mineralocorticoids alone do not induce a proinflammatory or profibrotic response in human proximal tubular cells unless co-incubated in a high sodium environment, suggesting the requirement of a specific co-factor or alteration in the cell status is required.

It may be that the high sodium environment promotes intracellular oxidative stress as the cell attempts to respond to the increased osmotic load, potentially via increased expression and activation of the NHE3 receptor.

To expand upon these findings there are a number of additional studies I would perform to confirm my findings and focus on the mechanisms of effect. Firstly, I would measure the osmolarity of the media serially over time after the addition of sodium in order to ascertain how quickly the cells adapt and I would also add a control well with an alternative osmotic agent e.g. dextrin. Secondly I would perform proliferation and apoptosis assays at each of the time points, in order to control for the effects of cell number. Thirdly I would attempt to quantify collagen 1 production via sonicated Sirius Red assays, as Western blotting has proven unreliable. Next, I would ascertain the effects of adding an ERK inhibitor (U0126) to the assay to confirm whether ERK signalling is involved. It would also be interesting to measure superoxide activity as a measure of oxidative stress and assess the effects of N-

acetylcysteine or tempol on collagen 1 gene expression in Aldo/sodium treated cells. It would be useful to study the role of intracellular regulatory pathways including SGK1. SGK1 promotes ENaC phosphorylation and increases the probability of ENaC being open, promoting sodium retention. It also promotes Nedd 4-2 phosphorylation, inhibiting Nedd 4-2:ENaC binding, thus preventing channel internalisation and degradation.

There are a number of limitations to this study. It would have been optimal to quantify the production of the collagen 1 protein, in order to confirm that increased gene expression was translated into protein formation. Also, whilst HK-2 cells respond to Aldo, it would be useful to confirm these findings in a more traditional Aldo target cell i.e. the distal tubular epithelium. Studies are however limited by the lack of an appropriate human distal tubular cell line or available primary renal tissue. The lack of a pro-inflammatory response seen may simply reflect the cytokines measured and perhaps a panel approach to cytokine measurement, allowing a wider array of cytokines to be tested, might have been of use. Also, the ELISAs used required quite substantial dilutions of cell culture supernatant which might have introduced error. The lack of a positive control for TGFB1 production by HK-2 cells means that it cannot be excluded that these cells simply do not produce TGFB1. Lastly, studies have shown conflicting effects of Aldo on cell proliferation and apoptosis and it would have been beneficial to determine its effects, and indeed those of sodium, on HK-2 cells under my study conditions.

8 Chapter Eight: General discussion

8.1 Summary of findings

The aims of this thesis were to assess mineralocorticoid status in patients with chronic kidney disease and ascertain whether levels of these molecules, in association with sodium status, were associated with negative prognostic features. The main findings were:

- A novel relationship exists between urinary sodium and urinary mineralocorticoid excretion in patients with CKD. High levels of urinary THDOC and THALDO excretion are associated with high urinary sodium excretion.

- Urinary THDOC excretion is independently associated with LVMI and THALDO excretion is independently associated with proteinuria in patients with CKD.

- Factors associated with LV mass differ between patients with CKD and patients with EH.

- Vascular function is not directly associated with mineralocorticoid status in patients with CKD.

- Excretion of urinary sodium, normalised to urinary creatinine, is associated with an increased mortality risk but not RRT risk in patients with CKD.

- Human proximal tubular cells in a high sodium environment respond to steroid stimulation by increasing collagen 1 gene transcription, a response which is inhibited by blockade of the mineralocorticoid receptor.

8.2 Strengths of these studies

Patients recruited to the clinical studies were very closely phenotyped. The usage of CMR enabled the most accurate determination of left ventricular mass available. Measurement of urinary steroid metabolites is the most accurate reflection of 24h steroid status, removing the inaccuracies of a spot plasma sample, and has not previously been reported in patients with CKD. The usage of these two methods in particular and a comparator group of patients with EH, provide a unique opportunity to study the regulation of MC production and interactions between steroids, the kidney, the heart and vasculature in patients with CKD. This, in the context of the laboratory and retrospective cohort studies, has resulted in a number of novel findings which deserve further attention.

8.3 Sodium excretion in CKD

In order to understand the interaction between mineralocorticoids and sodium in patients with CKD, it was important to study sodium excretion in these patients. Historically patients with uraemia have been shown to have elevated total body sodium. Chapter 3 demonstrated in a large cohort with extensive prospective follow-up a number of key findings. Firstly, urinary sodium excretion falls with advancing renal failure, but only once patients reach CKD 5. The kidney compensates for declining nephron mass by increasing fractional excretion of sodium, presumably via increased single nephron GFR. Whether patients reduce dietary sodium intake with advancing renal disease or whether the kidney loses its ability to excrete the sodium load, resulting in increased total body sodium is unclear. What was shown however is that urinary sodium excretion, when normalised to urinary creatinine, was an independent predictor of mortality but not of requirement for RRT, suggesting that the negative effects of sodium may be more related to cardiac or vascular effects rather than direct renal effects. Adjustment for urinary creatinine likely compensates for muscle mass and loss of tubular secretion with advancing CKD. This association with mortality has not previously been shown in a CKD population and lends support to the need for long-term trials assessing the benefits of dietary sodium reduction in CKD.

8.4 Mineralocorticoids in CKD

Chapter 4 describes a number of novel associations which address the ambiguity regarding mineralocorticoid status in CKD. Absolute levels of mineralocorticoid excretion did not differ between patients with EH and patients with CKD. It had previously been thought that aldosterone levels increase in CKD, for reasons that remain inadequately explained. This assumption largely derives from historical cohorts and relates to very low levels of renal function, rather than patients with CKD 3 or 4. It has however been shown in this thesis that in a cross-sectional cohort of patients with CKD3 or 4, aldosterone levels do not correlate with level of renal function. Similarly, levels were not higher in patients with diabetes. It should be noted however that CKD patients are presumed to have intravascular volume expansion and had significantly higher plasma potassium, therefore although steroid levels are not increased, this may be inappropriate as they should perhaps be suppressed if normal physiological principles applied.

Despite similar levels of plasma and urinary steroids in EH and CKD, physiological principles appear to be unexpectedly altered in patients with CKD. Strikingly, the strongest determinant of urinary THALDO and THDOC excretion in these patients was urinary sodium excretion, a reflection of dietary sodium intake. This relationship was not seen in patients with EH. Furthermore, plasma renin concentration (a surrogate for angiotensin II), cortisol metabolite excretion (a surrogate for ACTH) and serum potassium, all traditional regulators of mineralocorticoid synthesis and excretion, were not significantly associated with urinary steroid excretion in CKD.

Sodium and aldosterone are intrinsically linked – sodium is responsible for intravascular volume status. Reduced intravascular volume stimulates aldosterone production via the renin-angiotensin system. In the distal tubule aldosterone acts via the MR to stimulate ENaC opening, promoting sodium retention; and to a lesser degree via the NaH exchanger, with hydrogen ion loss and sodium retention. Concurrently the ROMK channels extrude potassium. Are the findings described in chapter 4 a reflection of altered aldosterone action in the distal tubule of CKD, or is steroid production by the adrenal cortex altered in CKD? Deeming the relationship to be a factitious reflection of renal leak seems unlikely and the size of the THALDO molecule means it should be freely filtered at the glomerulus.

Our studies show that high THALDO excretion is associated with high urinary potassium excretion, supporting the idea that aldosterone is still acting in the distal tubule in its traditional role. Alteration of ENaC function, resulting in sodium being excreted rather than retained in response to aldosterone, would seem unlikely, in that this would affect the electrochemical gradient in the tubule. It therefore seems more likely that the abnormality relates to the adrenal cortex and the stimuli or environment for aldosterone production.

It is possible that dietary sodium intake overwhelms renal sodium excretion in patients with CKD. Even a very small excess of intake would over time result in very significant sodium overload. Non-osmotic storage in the lymphatics is possible, however the likelihood is that sodium overload promotes intravascular volume overload, impaired baroreceptor sensitivity and loss of sympathetic inhibition of aldosterone release. Sodium overload also likely promotes oxidative stress. This, in the context of a uraemic milieu and mild acidosis, may alter sensitivity of the adrenal cortex to its usual stimuli, promoting aldosterone release perhaps in an attempt to normalise pH or potassium.

An alternative is that other stimuli such as endothelin take on a more prominent role in CKD. ANP is an inhibitor of aldosterone secretion, but levels of ANP increase in CKD. Or perhaps aldosterone and deoxycorticosterone production from extra-adrenal sites (such as the heart and kidney) increases in CKD and is regulated differently, but plasma aldosterone levels fall to almost zero post-adrenalectomy, so it is unlikely that we are detecting extra-adrenal synthesis. Therefore this is a novel relationship which remains unexplained and deserves further study (Figure 8-1).

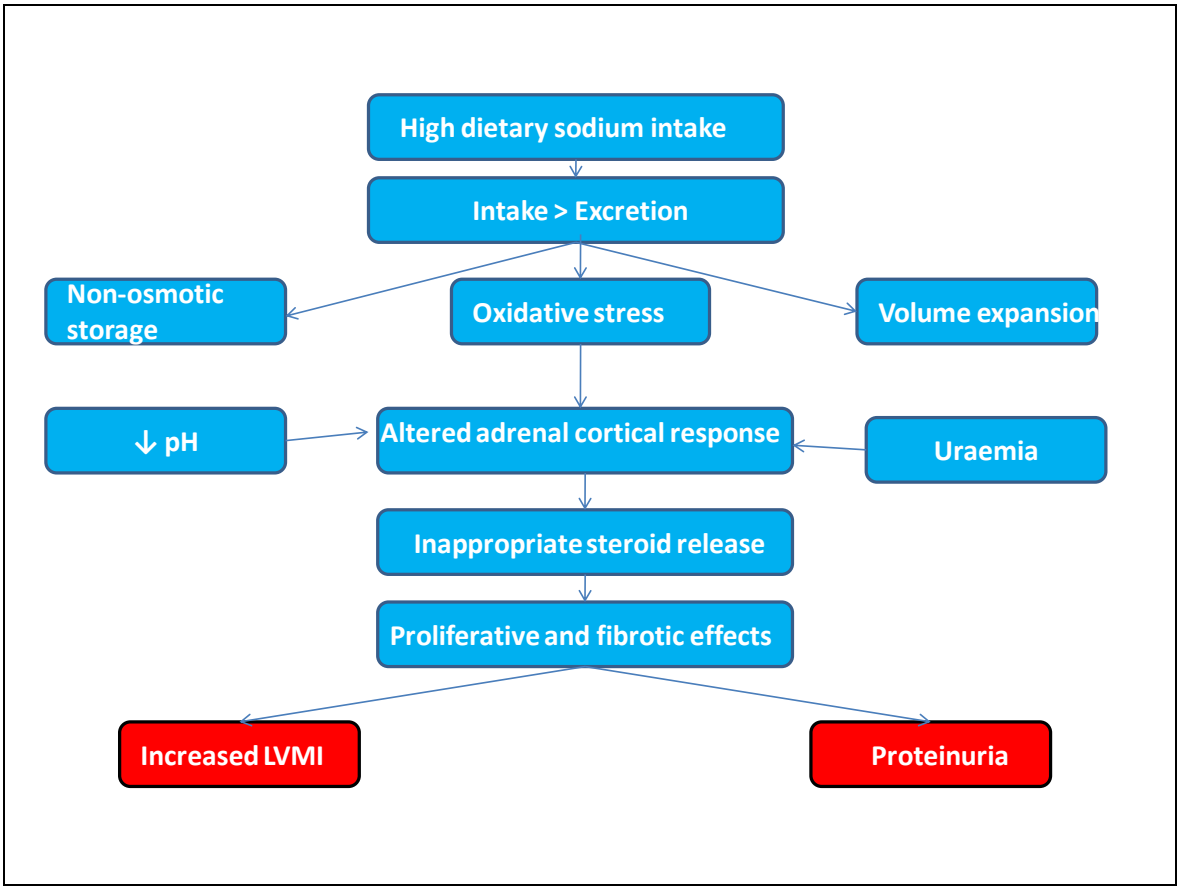


Figure 8-1: Potential relationship between sodium, steroids and negative effects (highlighted in red).

8.5 Adverse effects of mineralocorticoids

Regardless of the determinants of mineralocorticoid synthesis, we demonstrated that absolute levels of tetrahydrodeoxycorticosterone excretion were independently associated with LVMI (chapter 5) and tetrahydroaldosterone excretion with proteinuria excretion (chapter 4) – both significant predictors of mortality in the CKD population. These associations have not previously been reported. A synergistic interaction between steroids and sodium, such as has been reported in animal studies, was seen. This is of importance in that present methods of addressing cardiovascular risk and the elevated mortality associated with chronic kidney disease are inadequate. These findings support the usage of MR blockade, the only intervention found to reduce LV mass in CKD, in patients with CKD to reduce cardiovascular and renal risk and this should be the subject of large clinical trials.

Chapter 6 demonstrated that mineralocorticoids do not appear to be directly associated with vascular function in patients with CKD or patients with EH. This is surprising given that the vascular endothelium expresses the MR, that aldosterone is known to have direct effects including vasoconstriction and that MR blockade was shown to reduce pulse wave velocity. It may be that due to the 'real world' conditions of the study, the influence of mineralocorticoids on the vasculature (large or small vessel) could not be seen due to concomitant medications. An association was seen between high urinary sodium and poor endothelial function in patients with CKD, strengthening the evidence of an independent negative effect of sodium.

Chapter 7 extends the extensive work done demonstrating pathological effects of aldosterone. In human proximal tubular cells, MR binding, regardless of ligand, resulted in increased collagen 1 gene expression, but and only in a high sodium environment. The effects were blocked by an inhibitor of the MR, confirming the involvement of the MR. This finding supports previous studies highlighting a profibrotic role for aldosterone and MR receptor binding, whilst extending these findings to include a novel cell line under differing sodium conditions.

8.6 Limitations of these studies

There are limitations in these studies. Cross-sectional studies cannot assume causality and associations can only be reported as such. The small patient groups limit extensive

subgroup analysis and limit identification of relationships such as that between steroids, sodium and LV mass. Patients were not advised to discontinue their usual medications and as such drug effects may alter or disguise relationships. Urinary sodium intake was determined by 24h excretion. This is widely accepted as the gold standard measure of dietary sodium intake, however it is not a measure of whole body sodium. 24h blood pressure monitoring was undertaken in a minority and was closely related to clinic blood pressures, however the majority of patients were reluctant to undertake this investigation in addition to the 24h urine collection. GFR was estimated using validated equations, however measurement of true GFR would have been useful. Similarly, we measured total proteinuria rather than albuminuria and this additional information may have provided further insight.

8.7 Future studies

A physiological study of adrenal aldosterone response to traditional stimuli of AgII, ACTH and potassium infusion in patients with CKD off medication would be beneficial. It would also be interesting to confirm whether or not the normal diurnal variation in aldosterone levels, a reflection of ACTH, exists in patients with CKD.

In order to interpret the steroid abnormalities further, it would be useful to have an objective assessment of body fluid status, for example utilising bioelectrical impedence analysis. A measure of total body sodium, for example via MRI ^{23}Na spectroscopy or ^{24}Na exchangeable sodium, would also inform the interpretation of whether patients with CKD are chronically sodium overloaded and whether this relates to adrenal steroid release in the same way that urinary sodium excretion does. Assessment of markers of oxidative stress and WNK production and correlation with measures of sodium status and aldosterone production would also be of interest.

The finding that determinants of LV mass differ between patients with CKD and patients with EH suggest that it would be worthwhile expanding the cohort to include patients with less advanced renal impairment to establish at what stage these differences develop. DOC excretion associated more closely with LVMI than Aldo in patients with CKD, suggesting that further study of this mineralocorticoid, which has been largely overlooked until now, is warranted. What effects does DOC have on the myocardium?

A comparison of EndoPAT and brachial artery flow mediated dilatation in patients with CKD would be useful in order to validate the technique more thoroughly in patients with CKD.

All patients recruited into the study consented to longitudinal follow-up and it will be of interest to determine whether steroid excretion rates correlate with renal and patient survival and cardiovascular events.

Follow-up studies to the laboratory work are extensively discussed in chapter 8. Further to this, in vitro assessment of adrenal cortical cells cultured in a high sodium ‘uraemic’ milieu would be of interest in terms of aldosterone and deoxycorticosterone production.

8.8 Conclusions

- Urinary steroid excretion, an integrated measure of steroid status, is not elevated in moderate CKD as compared with patients with EH.

- Patients with diabetic nephropathy do not exhibit RAAS overactivation, compared with primary renal diseases.

- Determinants of urinary steroid excretion differ between patients with CKD and patients with EH, suggesting that stimulation of aldosterone production differs in the context of uraemia.

- Around half of patients with CKD 3/4 have left ventricular hypertrophy despite reasonable blood pressure control.

- Urinary tetrahydrodeoxycorticosterone excretion, in addition to male gender and systolic blood pressure, is significantly associated with LVMI, unlike in EH where plasma aldosterone concentration is the significant independent predictor.

- Urinary tetrahydroaldosterone excretion is independently associated with proteinuria excretion in patients with CKD.

- An interaction was seen between high urinary steroid excretion, high urinary sodium excretion and proteinuria and LVMI.

- Pulse wave velocity is higher in patients with diabetic nephropathy and is predominantly determined in CKD by pulse pressure, age and waist circumference.

- Urinary sodium excretion was associated with endothelial dysfunction in patients with CKD and pulse wave velocity in patients with EH, supporting a negative role for sodium in the vasculature.

- A high urinary sodium:creatinine ratio is independently associated with risk of death in patients with CKD.

- Co-culture of human proximal tubular cells with aldosterone and sodium is associated with upregulation of collagen 1 gene synthesis, an effect which is inhibited by blockade of the mineralocorticoid receptor.

- These studies support a role for mineralocorticoids in the adverse phenotype seen in CKD and suggest that further study of MR ligands and blockade should be undertaken.

9 Appendix

9.1 Publications

Publications containing work undertaken for this thesis:

McQuarrie EP, Patel RK, Mark PB, Delles C, Connell J, Dargie HJ, Steedman T, Jardine AG. Association between proteinuria and left ventricular mass index: a cardiac MRI study in patients with chronic kidney disease. Nephrol Dial Transplant 2011; 26(5):1563-9.

McQuarrie EP, Stevens KK, Sands W, Hillyard DZ, Patel RK, Mark PB, Jardine AG. Fibroblast growth factor 23 predicts left ventricular mass and induces cell adhesion molecule formation. Int J Nephrol 2011; Epub Aug 9.

Publications related to the work in this thesis:

Patel RK, Jardine AG, Mark PB, Cunningham AF, Steedman T, Powell JR, McQuarrie EP, Stevens KK, Dargie HJ, Jardine AG. Association of Left Atrial Volume With Mortality Among ESRD Patients With Left Ventricular Hypertrophy Referred for Kidney Transplantation. Am J Kidney Dis. 2010 Jun;55(6):1088-96.

Patel RK, Oliver S, Mark PB, Powell JR, McQuarrie EP, Traynor JP, Dargie HJ, Jardine AG. Determinants of left ventricular mass and hypertrophy in hemodialysis patients assessed by cardiac magnetic resonance imaging. Clin J Am Soc Nephrol. 2009 Sep;4(9):1477-1483.

9.2 Supplementary material

1. Patient consent form
2. Patient information sheet
3. GP letter regarding patient participation.

CONSENT FORM

Please initial each box to show that you have read and understood each point.

1. I confirm that I have read and understand the information sheet dated 17.11.2008, version 2.0, for the above study, that I have had the opportunity to ask questions and that I have received satisfactory answers to the questions that I have asked ☐

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 sections of any of my medical notes may be looked at by responsible individuals from the North Glasgow NHS University Trust where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I understand that personal information about me including information about my health will be collected and processed as part of the study and I consent to this. ☐

5. I understand that data about me as collected for this study including information about my health may be passed to other members of the Study team. I understand that information that may identify me will be removed before any data leaves the (Western Infirmary, Glasgow). I give my consent for this to happen. ☐

6. I understand that blood and urine will be frozen and stored in Glasgow as required for further analysis in relation to this study and I give my consent for this to happen. ☐

7. I consent to my blood being stored for genetic testing. This will be anonymous and I will not be identified. ☐

8. I understand that my G.P will be informed that I am taking part in this study. I give my consent for this to happen. ☐

9. I agree to take part in the above study. ☐

_____	_____	
Name of Patient	Signature	Date
_____	_____	
Name of Person taking consent (if different from researcher)	Signature	Date
_____	_____	
Researcher	Signature	Date

Patient information sheet

Renal Research Group
British Heart Foundation Cardiovascular Research Centre /
Western Infirmary
University of Glasgow, G12 8TA

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you decide to take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Please take your time to decide whether or not you wish to take part.

Part 1

What is the purpose of the study?

Factors which predict the progression of kidney disease include high blood pressure (hypertension) and protein in the urine (proteinuria). The role of aldosterone (a hormone produced by the adrenal gland) in hypertension has become appreciated in the last decade. Our principal research question is whether the level of aldosterone produced by the body influences kidney damage and hence progression of kidney disease, and whether this influence is via mechanisms related to salt intake and proteinuria. We would also like to study the relationship between the stiffness of the blood vessels in the circulation and aldosterone levels, proteinuria and outcome.

Why have I been chosen?

You have high blood pressure and normal kidney function or you have kidney disease secondary to diabetes, IgA nephropathy or membranous nephropathy.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You will still be free to withdraw at any time in the future and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care that you receive.

What will happen to me if I take part?

If you take part, you will be asked to undertake a series of tests. These all take place on the one study visit. Following this study visit no further tests are required but we will study your progress during your usual follow up visits via the renal clinic electronic patient record (EPR) which is already routinely used for standard out patient appointments. The detailed study visit takes about 2 hours in total. If required we will provide transport to and from your home for the visit.

What do I have to do?

Study Visit

You will be asked to have the following tests: Blood pressure measurement, weight, height and waist measurement, a physical examination and a blood test (this will be taken from your arm in the usual fashion). Approximately 40 mL of blood will be taken equating to three tablespoons. These blood samples will be for analysis of aldosterone and related hormones in the blood. Like any other blood test this may cause a little discomfort and occasionally bruising. You will be asked questions about the drugs you are currently taking and your medical history. We will perform an electrocardiograph to look at your heart. This will take 5 minutes and involves stickers being placed on your chest and arms and a recording taken.

- We would like to examine the <u>stiffness of your arteries</u>.

  For this purpose we will measure how fast the pulse travels through your body from the heart to the groin. This involves measurement of the pulse with a pencil-like probe which is placed to your neck and to your groin for each about 1 minute. In parallel we will look at the electrical activity of your heart with an electrocardiograph.

- We would like to examine the consequences of stiff arteries for your heart. This examination again involves assessment of your pulse with a pencil-like probe. It is carried out at the artery at your wrist and takes 1 to 2 minutes. At the same time we will measure your blood pressure with a cuff as known to you from your GP. We can then calculate the pulse waveform and the blood pressure in the large arteries close to your heart.
- We would like to examine the function of your arteries.
  For this test we use again the pencil-like probe to measure the pulse at your wrist. We will perform a few readings to make sure that the signal is stable. You would then receive two puffs of glycerol trinitrate (GTN) spray under your tongue. This spray makes blood vessels wider and is therefore commonly used to treat chest pain. We will measure changes in the pulse waveform in response to this test. You would then inhale two puffs of salbutamol spray which may be known to you as a spray against asthma and chronic bronchitis. GTN spray can cause a headache after it is given, and lower the blood pressure. Salbutamol can cause a temporary tremor. Again we will measure the pulse waveform. Because we must be sure to have stable signals both before and after administration of the sprays this part of the study may take up to 30 minutes.
  Alternatively we may examine blood flow in your fingers. We will use a standard blood pressure cuff to block the blood flow to your forearm for 5 minutes. This may cause some numbness of your forearm which disappears shortly after release of pressure of the cuff. Blood flow in your fingers will be measured before, during and after the 5-minutes block with probes on your left and right index finger. This test takes about 25 minutes in total. In patients with blood vessel disease of their arms (*e.g.* Raynaud's disease) we would not perform the test involving blocking blood flow to the arm for 5 minutes.
- We would like to assess your heart function with a MRI scanner - CMR Scan – this involves lying on a table as it passes through a large circular magnetic resonance scanner. This does not involve any X-rays. However, the scan may be noisy and may rarely cause claustrophobia. Your blood pressure will be measured during the scan which will take approximately 25 mins in total.

- Both before and following these tests we will ask you to perform a 24 hour collection of urine. You will receive containers to collect your urine specimens and can leave to return the following day with the sample.
- We will also ask you to wear a blood pressure monitor for 24 hours at home. This involves wearing a blood pressure cuff around your arm and a small portable recorder on your belt. You should go about your daily activities as normal, except showering or taking a bath whilst wearing it. The monitor takes your blood pressure every 20 minutes and hourly overnight. **Your sleep may be disrupted as the cuff inflates**. This method gives us a very accurate measure of your normal blood pressure.
- Following this visit no further specific study visits are required but we will continue to monitor you blood pressure, weight and kidney function when you routinely attend the out patient clinic.

Your doctor will be informed of your participation in this study.

What are the possible disadvantages and risks of taking part?

We have taken every step in the design of this study to minimise any possible disadvantages and risks. We will take blood samples and perform intravenous cannulation which can cause bruising and swelling and may be slightly painful. We will do our utmost to ensure that the urine collection is collected at a time that is most convenient for you

What are the possible benefits of taking part?

The study may not benefit you directly, but the information we receive may help us to understand mechanisms involved in progression of kidney disease.

What happens when the research study stops?

We would ask your permission to store your blood and urine samples (in anonymised form) in the University of Glasgow so that we can consider them for use in future research studies that we may carry out. (Any future use of the samples would require the approval of a Research Ethics Committee for that project).

Part 2

What if relevant new information becomes available?

If any clinically significant information comes to light as a consequence of taking part in this study, we will inform your GP (with your permission).

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any time without having to give a reason. However, we would ask your permission to use any information collected during the time of your participation for research purposes. Any stored blood or urine samples that can be identified as yours will be destroyed if you wish.

What if there is a problem?

Complaints: If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. In the event of a complaint contact the NHS:

Complaints Office,

Western Infirmary,

Dumbarton Road,

Glasgow G11 6NT

Tel: 0141 211 2257 or 2926

Harm: In the event that something does go wrong and you are harmed during the research study, there are no special compensation arrangements. If you are harmed and this is due to someone's negligence, then you may have grounds for a legal action for compensation against the University of Glasgow or NHS Research and Development, but you may have to pay for your legal costs. The normal National Health Service complaints mechanism will still be available to you.

Will my taking part in this study be kept confidential?

Yes. All information which is collected about you during the course of the research will be kept strictly confidential. It may have been your GP who contacted you about this study initially, but if we contacted you directly throught the hospital clinic, we would like to inform your GP of your involvement in the

study and will ask for your permission to do this. All other information about you which leaves the hospital will have your name, address and date of birth removed so that you cannot be recognised from it.

What will happen to any samples I give?

A portion of the blood samples will be analysed immediately by the Department of Biochemistry at Gartnavel Hospital. The remainder of the blood samples, along with the urine samples, will be transferred to the University of Glasgow for analysis. The samples will be analysed in the University of Glasgow by various biochemical techniques in order to measure the levels of certain 'markers'. The samples will be anonymised so that you will not be identifiable from them.

Will any genetic tests be done on the samples that I give?

Yes we will use some of your blood sample for genetic testing. Following this current study, we would ask your permission to store any remaining samples so that we may consider them for use in future research studies that we may carry out (if a local Ethics Committee deems the study appropriate). Genetic samples will be stored in a coded fashion, to prevent identification of samples by third parties. Genetic samples will not identify individual patients. Any results from the planned or future genetic studies will not have any healthcare implications for you and hence we would not normally feed these results back to you.

What will happen to the results of the current research study?

The results of this study will be published in medical journals, reports and textbooks. You will not be identifiable in any publication or report. The reports will be stored for 6 years at The University of Glasgow.

Who is organising and funding the research?

The research is being organised and sponsored by the University of Glasgow.

Who has reviewed the study?

This study has been given a favourable ethical opinion for conduct in the NHS by West Glasgow Research Ethics Committee.

Contact details

You may contact me (one of the Principal Researchers) directly by telephoning 0141 211 1867 for further information at any time. Alternatively, you may contact Dr Neal Padmanabhan, Consultant Nephrologist who is acting as an independent advisor – contact 0141 211 2178.

Many thanks for taking the time to read this information.

Dr Patrick Mark
Department of Medicine
The University of Glasgow

GP letter

Aldosterone and progression of renal disease

Investigators – Prof J Connell, Prof A Jardine

British Heart
Foundation Cardiovascular Research Centre
126 University Place
Glasgow
G12 8TA

Dear Doctor,
Re:
Name
Date of Birth

Your patient attended today to take part in a study to evaluate markers of progression of renal disease. They were recruited from the renal clinic at the Western Infirmary. The study involves taking basic anthropometry, urine and blood samples. These are for baseline biochemistry and novel markers. Blood is taken for DNA analysis, which is anonymised. They also underwent tests to look at endothelial function, pulse wave analysis and cardiac MR scanning.
As these tests are predominantly for research purposes we do not plan to send a report of these. However, should you wish to obtain further information please do not hesitate to contact myself, Professor Jardine or Professor Connell on 0141 330 2723.
Yours sincerely,
Dr. Emily McQuarrie
Clinical Research Fellow
BHF CVRC

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