The Role of DDAH and ADMA in Kidney Disease

A thesis presented for the degree of Doctor of Philosophy by

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DECLARATION OF ORIGINALITY

I, James Alexander Patrick Tomlinson confirm that the work presented in this thesis is my own. Where information has been derived from outside sources or data obtained by others, I can confirm this has been indicated in the text.

James Tomlinson
October 2013

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Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide (NO) synthesis and elevated plasma levels associate with poor cardiovascular and renal outcomes. The dimethylarginine dimethylaminohydrolase enzymes (DDAHs; 1 and 2) metabolise ADMA. A DDAH1 gene variant associates with higher kidney tissue mRNA expression, lower plasma ADMA but counter-intuitively, a steeper rate of eGFR decline. This indicates that renal DDAH1 activity may be deleterious and circulating ADMA does not necessarily reflect the NO-ADMA balance (or severity of disease) within kidney tissue. This study tests the hypothesis that reduced renal DDAH1 activity protects against the progression of kidney function decline, independent of circulating ADMA.

Renal DDAH1 expression predominates within the proximal tubule. A novel proximal tubule-specific DDAH1 knock-out (PTD1KO) mouse was developed, which demonstrated tubule-specific dysregulation of ADMA and NO that was not evident systemically. Phenotyping studies in PTD1KO mice did not identify consistent alterations of urinary biochemistry at baseline or after salt loading, however, proteomic analysis revealed significant alterations of urinary peptides at baseline; including down-regulation of uromodulin and collagen. At 12 weeks following folate renal injury, the PTD1KO mouse exhibited less kidney function decline, collagen deposition and pro-fibrotic gene expression (Col12α, TGFβ and ET-1) than controls. Furthermore, ADMA and DDAH1 inhibition reduced tubular sodium and fluid reabsorption in rat microperfusion studies, although studies in PTD1KO mice failed to reproduce this effect. Finally, in vitro studies using a PT cell line and primary PT culture indicated an inhibitory effect of ADMA upon PT cell proliferation.

Consistent with recent human genetic studies, these data provide experimental evidence indicating a reduction of renal tubule DDAH1 activity can protect against progressive kidney fibrosis and function decline, independent of plasma ADMA. This work provides novel insights into the role of the NO-ADMA-DDAH axis within the kidney, particularly the tubule.
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Jill was incredibly patient during my initial few months in the lab and provided me with a sound introduction to cell culture and how to properly design experiments. She was always available to help and discuss issues, no matter how trivial – despite being targeted by a large number of colleagues for similar support.

James, my formal supervisor, was inspirational with his passionate approach towards physiology and remarkable ability for asking the right questions; providing a seemingly relentless stream of tantalising hypotheses to test. James’ supervision in the lab and company over a pint have been both productive and a real pleasure. The members of James’ laboratory group were all welcoming and great to work with. The individual and collective benefits of working as part of a team - sharing ideas and workload are difficult to quantify but should not be underestimated.

This project would not have taken shape without Ben’s input. Besides generating the initial hypothesis, he provided guidance through many aspects of this PhD. His academic passion and impressive hungry pursuit of his own career path has been inspirational.

True for anything I have achieved in life, I owe my parents loving gratitude for their unconditional support and positive influence.
Finally Lina, whom I married during this work. She has closely witnessed the natural history of a PhD project with all of its highs and lows. Despite many spoiled weekends, she has provided insightful moral support and encouragement throughout, not to mention countless gastronomic rewards for extended shifts of staring at a computer screen.
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<tr>
<td>αSMA</td>
<td>Alpha-smooth muscle actin</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenine diphosphate</td>
</tr>
<tr>
<td>ADQI</td>
<td>Acute Dialysis Quality Initiative</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin II receptor blocker</td>
</tr>
<tr>
<td>ACEi</td>
<td>Angiotensin converting enzyme inhibitor</td>
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<td>C57 black 6 strain</td>
</tr>
<tr>
<td>CAT</td>
<td>Cationic amino acid transporter</td>
</tr>
<tr>
<td>CCL2</td>
<td>Chemokine ligand-2</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribose nucleic acid</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CKD-MBD</td>
<td>Chronic kidney disease-mineral bone disorder</td>
</tr>
<tr>
<td>Col12α</td>
<td>Collagen type 1 subunit alpha-2</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CᵥqPCR</td>
<td>qPCR fluorescence threshold</td>
</tr>
<tr>
<td>CV</td>
<td>Cardiovascular</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>D1</td>
<td>Floxed DDAH1 transgene</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DDAH</td>
<td>dimethylarginine dimethylaminohydrolase</td>
</tr>
<tr>
<td>ddH2O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DGV</td>
<td>dimethylguanidino valeric acid</td>
</tr>
<tr>
<td>DMA</td>
<td>dimethyl amine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribose nucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphates</td>
</tr>
<tr>
<td>DOCA</td>
<td>deoxyxorticosterone acetate</td>
</tr>
<tr>
<td>ECAR</td>
<td>Extracellular acidification rate</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EDV</td>
<td>endothelium-dependent vasodilatation</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>eGFR</td>
<td>estimated glomerular filtration rate</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial NOS/NOSIII</td>
</tr>
<tr>
<td>epPCR</td>
<td>End-point polymerase chain reaction</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic cell</td>
</tr>
<tr>
<td>ESRD</td>
<td>end stage renal disease</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FAM</td>
<td>6-carboxyfluorescein</td>
</tr>
<tr>
<td>FCCP</td>
<td>carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FeNa</td>
<td>Fractional excretion of sodium</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>FXR</td>
<td>farnesoid X receptor</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GN</td>
<td>glomerulonephritis</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HPRT</td>
<td>hypoxanthine phosphoribosyltransferase 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NHPSTs</td>
<td>Normal human proximal straight tubular cells</td>
</tr>
<tr>
<td>NKCC2</td>
<td>Sodium-potassium chloride co-transporter</td>
</tr>
<tr>
<td>NKF</td>
<td>US National Kidney Foundation</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NOx</td>
<td>Nitrogen oxide species</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal NOS/NOSI</td>
</tr>
<tr>
<td>NRF</td>
<td>Nuclear factor</td>
</tr>
<tr>
<td>ns</td>
<td>Non-significant</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatories</td>
</tr>
<tr>
<td>NTN</td>
<td>Nephrototoxic nephritis</td>
</tr>
<tr>
<td>NTS</td>
<td>Nephrotoxic serum</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAH</td>
<td>Para-aminohippurate</td>
</tr>
<tr>
<td>PAN</td>
<td>Puromycin aminonucleoside nephrosis</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid-schiff</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCr</td>
<td>Plasma creatinine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PRMT</td>
<td>Protein methyl transferases</td>
</tr>
<tr>
<td>PT(C)</td>
<td>Proximal tubule (cell)</td>
</tr>
<tr>
<td>PTD1KO</td>
<td>Proximal tubule specific DDAH1 knock-out</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>RIFLE</td>
<td>Risk; Injury; Failure; Loss; End-stage renal disease</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio-Immunoprecipitation Assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute (cell culture media)</td>
</tr>
<tr>
<td>RPOL</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-Kinase</td>
</tr>
<tr>
<td>RPL13A</td>
<td>Ribosomal protein 13A</td>
</tr>
<tr>
<td>RRT</td>
<td>Renal replacement therapy</td>
</tr>
<tr>
<td>rtPCR</td>
<td>Reverse transcription PCR</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic BP</td>
</tr>
<tr>
<td>SCX</td>
<td>Strong cation exchange chromatography</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDMA</td>
<td>Symmetrical dimethylarginine</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>small inhibitory RNA</td>
</tr>
<tr>
<td>SNAP</td>
<td>S-nitroso-N-acetyl-L,L-penicillamine</td>
</tr>
<tr>
<td>SNO-Hb</td>
<td>S-nitrohaemoglobin</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>SUN</td>
<td>Serum urea nitrogen</td>
</tr>
<tr>
<td>SVR</td>
<td>systemic vascular resistance</td>
</tr>
<tr>
<td>TAL</td>
<td>Thick ascending limb</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF</td>
<td>Tubuloglomerular feedback</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>UCL</td>
<td>University College London</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
</tr>
<tr>
<td>UMOD</td>
<td>uromodulin</td>
</tr>
<tr>
<td>UNx</td>
<td>uninephrectomy</td>
</tr>
<tr>
<td>UUO</td>
<td>Unilateral ureteric obstruction</td>
</tr>
<tr>
<td>UV</td>
<td>Utero-vaginal</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 The kidney in health

1.1.1 Physiological kidney function

The central role of the mammalian kidney is to maintain an internal metabolic balance by regulating circulatory volume and systemic blood pressure whilst removing unwanted solutes. The human kidney is highly vascular and despite weighing an average of only 150 grams, it can filter up to 150 litres of blood per day [1] (Figure 1A). Each kidney contains 1-2 million functional subunits [2] (nephrons; Figure 1B) that interface with the intravascular and urinary compartments in order to monitor and regulate the composition and volume of circulating plasma. To achieve this, three processes are key; 1) filtration of plasma by the glomerulus (ensuring large plasma proteins remain), 2) selective reabsorption of filtrate into the tubular epithelial cell lining and finally, 3) secretion of unwanted solutes and metabolites from peritubular capillaries into the tubular fluid.

![Figure 1. Schemata of the kidney and nephron](Image)

A) Whole kidney structure; a protective fibrous capsule envelopes the kidney; cortex houses glomeruli and tubular portions of the nephron; medulla contains Loops of Henle, collecting ducts and larger vessels; central pelvis contains the bundle of vessels, nerves and urinary collecting system. B) The nephron; blood is filtered at the glomerulus; ultrafiltrate passes into the proximal tubule where the majority is selectively reabsorbed back into the peritubular capillaries and other metabolites are secreted; further water and solute exchange occurs through the Loop of Henle (via vasa rectae capillaries) and distal tubule; urine flows down the collecting duct. (Images adapted from Wellcome Images, N0026029).
In addition to excretion, the kidney has other critical homeostatic responsibilities including secretion of the hormone erythropoietin (stimulating bone marrow production of red blood cells), and hydroxylation of vitamin D into its active form - essential to calcium and phosphorus regulation ensuring bone health.

1.1.2 Measurement of kidney function

Kidney function is determined by a quantification of the glomerular filtration rate (GFR). Creatinine, a metabolite of creatine phosphate from muscle, serves as a plasma marker of GFR. After release into plasma it is freely filtered at the glomerulus but since it does not undergo tubular reabsorption, it reflects GFR under steady-state conditions of dietary intake and muscle breakdown. Limitations to its interpretation include; the assumption of steady-state; individual variability of muscle mass; and variable tubular secretion of creatinine (up to 28% of total clearance [3]) resulting in over-estimates of GFR. Despite being an imperfect surrogate for GFR, creatinine remains the most common clinical marker of renal function, largely due to the convenience of automated plasma measurement [4, 5] whilst gold standard tests for measuring GFR using radioisotopes or inulin clearance remain impractical.

The majority of assays to measure creatinine are based on the original colorimetric Jaffe reaction [4], although the specific techniques used vary widely between laboratories which in turn affects their performance [6]. More recently, official working groups such as the National Kidney Disease Education Program (NKDEP, USA) have recommended calibration of clinical assays against the reference method – isotope dilution mass spectrometry [7].

To counter some of the limitations attributed to muscle mass, current clinical practice uses a derivation of plasma creatinine proposed by the Modification of Diet in Renal Disease study group, which corrects for confounding variables such as age, gender and ethnicity and provides a more precise value for many individuals [8]. Most laboratories now routinely provide estimated GFR (eGFR) values (alongside creatinine values) that are derived using a simplified MDRD equation (Figure 2 [9]).
A) Complete MDRD-1 equation (mL/min/1.73m²)

\[ 170 \times [PCr]^{-0.999} \times [age]^{-0.176} \times [0.762 \text{ if female}] \times [1.180 \text{ if black}] \times [SUN]^{-0.170} \times [Alb]^{0.318} \]

B) Simplified MDRD-2 equation (mL/min/1.73m²)

\[ 186 \times [PCr]^{-1.154} \times [age]^{-0.203} \times [0.742 \text{ if female}] \times [1.212 \text{ if black}] \]

Figure 2. MDRD equations for calculating GFR from serum creatinine values

(A) Complete; (B) Simplified. Where PCr=serum creatinine concentration (mg/dL) (alkaline picrate method); SUN=serum urea nitrogen concentration (mg/dL) (urease method); Alb=serum albumin concentration (g/dL) (bromocresol green method).

Despite widespread international use of serum creatinine and the MDRD equation, debate continues as to the best method to quantify GFR, given the limitations of creatinine. Alternative serum markers (such as cystatin C and symmetric dimethylarginine; SDMA, see further text [10]) or improved creatinine-based eGFR formulae such as that developed by the CKD Epidemiology Collaboration (CKD-EPI, [11]) have been proposed but as yet, have not been adopted in routine clinical practice.

Accurate measurement of GFR in experimental animals is not straightforward. Performed traditionally using inulin or para-aminohippurate (PAH), results can be confounded by anaesthetic agents and volume depletion resulting from sequential venesection. Similar to the clinical setting, concerns have been raised regarding the specificity of assays based on the Jaffe reaction in mice [12]. High pressure liquid chromatography (HPLC) of serum creatinine has been reported to be a much more reliable assay that correlates well with labelled inulin clearance [13] and more recently, fluorescein isothiocyanate (FITC)-labelled sinistrin (a polysaccharide similar to inulin) has been used as an injectable marker of GFR that can be quantified transcutaneously over time [14].
1.2 The kidney in disease

1.2.1 Mechanisms of kidney injury

1.2.1.1 Perfusion dependence

For their size (<2% of body mass), the kidneys require a relatively large proportion of cardiac output (~20%) in order to satisfy their metabolic demands. This presents a particular vulnerability to injury for if perfusion pressure is compromised for instance, by hypovolaemia; vascular disease (eg. hypertension or diabetic vasculopathy) or excess vasoactive hormones and drugs, then GFR declines as tubules quickly become hypoxic and incapable of selecting essential filtrate from waste.

Other functional and structural factors predispose the kidney to injury which are best described in terms of the nephron region affected: glomerular or tubulointerstitial.

1.2.1.2 Glomerular disease

Diabetes is the leading cause of chronic renal disease in the Western World and is characterised by progressive glomerular destruction [15]. Sustained hyperglycaemia causes micro-vascular disease within the glomerular capillary bed leading to failure of sieve function, proteinuria and eventually, irreversible glomerulosclerosis.

The glomerulus is particularly susceptible to immune-mediated damage that causes a significant proportion of acute intrinsic kidney injury. Local or remote inflammatory responses can precipitate glomerulonephritis due to trapping of circulating immune complexes within the small-diameter glomerular capillary loops. In addition, the glomerular basement membrane contains two antigens that cause specific types of renal disease; the Amyloid P component (a glycoprotein) and the Goodpasture antigen (associated with type IV procollagen).

Histological features of primary glomerular disease are classified according to:

1. Distribution of abnormal glomeruli (focal or diffuse).
2. Distribution of abnormalities within single glomeruli (global or segmental).

3. Nature of glomerular abnormalities (endothelial, mesangial or endothelial cell proliferation; basement membrane appearance; presence of immune complexes) [1]

1.2.1.3 Tubulointerstitial disease

The renal tubule is highly metabolically active and its vulnerability to hypoxia is compounded by a low physiological oxygen tension of blood reaching the deep medullary peritubular capillaries [16]. Active reabsorption of filtrate and secretion of unwanted solutes is not only energy-demanding, but concentrates metabolites or exogenous compounds locally, to levels that can cause direct cellular toxicity or simply overwhelm tubular cell function. In addition, many drugs (e.g. non-steroidal anti-inflammatories; NSAIDs) and antibiotics can directly trigger a tubulointerstitial hypersensitivity reaction, while compounds such as urate precipitate within tubular lumina causing obstruction, inflammation and cell death.

Finally and perhaps most significantly, the tubulointerstitium lies structurally and functionally between the glomerular and urinary collecting system and is therefore exposed to the migrating insults from glomerular protein leak upstream (Chapter 1.2.8) or ascending infection and obstructive pressure from downstream within the lower urinary tract.

Histological features of tubulointerstitial disease include:

1. Interstitial oedema
2. Inflammatory cell infiltration (lymphocytes, plasma cells, macrophages, neutrophils)

And later:

3. Interstitial fibrosis
4. Tubular atrophy
5. Persistent lymphocyte-macrophage infiltration [1]

In a clinical setting, the manifestations of renal damage fall into two categories defined by chronicity: acute kidney injury (AKI) and chronic kidney disease (CKD).
1.3  Acute kidney injury (AKI)

1.3.1  Definition and classification of AKI

Broadly defined as a rapid loss of renal function, *acute kidney injury* (AKI) replaced the term *acute renal failure* as part of an international definition and classification campaign initiated by the Acute Dialysis Quality Initiative (ADQI) [17]. This international collaboration produced the “RIFLE” criteria of AKI definition and staging based upon serum creatinine, urine output and the need for renal replacement therapy RRT (“RIFLE”: Risk; Injury: Failure; Loss; End-stage renal disease [17]). Subsequent formal collaborations have developed these principles (Acute Kidney Injury Network; AKIN [18]) and most recently, the Kidney Disease: Improving Global Outcomes group (KDIGO) published definition and staging guidelines that have been adopted by most countries including the UK Renal Association (Figure 3) [19].

AKI is defined with ANY of the following:

a) Increase in serum creatinine ≥0.3mg/dL (≥26.5µmol/L) within 48 hours
b) Increase in serum creatinine ≥1.5x baseline within 7 days
c) Urine volume <0.5mL/kg/h for 6 hours

<table>
<thead>
<tr>
<th>Stage</th>
<th>Serum creatinine</th>
<th>Urine output</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5-1.9 times baseline OR ≥0.3mg/dL (≥26.5µmol/L) increase</td>
<td>&lt;0.5mL/kg/h for 6-12 hours</td>
</tr>
<tr>
<td>2</td>
<td>2.0-2.9x baseline</td>
<td>&lt;0.5mL/kg/h for ≥12 hours</td>
</tr>
<tr>
<td>3</td>
<td>3.0x baseline OR ≥4.0mg/dL (≥353.6µmol/L) OR initiation of RRT OR in patients &lt;18 years, decrease in eGFR &lt;35mL/min/1.73m$^2$</td>
<td>Anuria for ≥12 hours</td>
</tr>
</tbody>
</table>

Figure 3. Definition and staging of Acute Kidney Injury (AKI)

As described by the KDIGO guidelines [19]

1.3.2  Incidence of AKI

The incidence of community acquired AKI is around 1% but rises significantly to 7% following hospital admission [20, 21]. The difference derives from a proportion of community AKI events inevitably passing undiagnosed. Furthermore, hospitalisation increases the likelihood of developing AKI due to consequences of the presenting condition plus exposure to iatrogenic insults
such as contrast- or drug-related nephrotoxicity. The recent NCEPOD report (National Confidential Enquiry into Patient Outcome and Death, 2009) stated that a fifth of all hospital-acquired AKI was both predictable and avoidable and only 50% of AKI care was considered by advisors as good [22]. For this reason, considerable efforts have been made to produce internationally standardised definitions for AKI and guidelines to assist its effective management [23, 24].

<table>
<thead>
<tr>
<th>Causes / exposures</th>
<th>Risk factors / susceptibilities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepsis</td>
<td>Volume depletion</td>
</tr>
<tr>
<td>Critical illness</td>
<td>Advanced age</td>
</tr>
<tr>
<td>Circulatory shock</td>
<td>Female gender</td>
</tr>
<tr>
<td>Burns</td>
<td>Black race</td>
</tr>
<tr>
<td>Trauma</td>
<td>CKD</td>
</tr>
<tr>
<td>Cardiac surgery</td>
<td>Chronic diseases (heart, lung, liver)</td>
</tr>
<tr>
<td>Major surgery (non-cardiac)</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>Nephrotoxic drugs</td>
<td>Cancer</td>
</tr>
<tr>
<td>Radiocontrast dyes</td>
<td>Anaemia</td>
</tr>
<tr>
<td>Poisonous plants / animals</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1. Causes and risk factors for AKI**
Adapted from KDIGO Clinical Practice Guideline for Acute Kidney Injury [25]

1.3.3 Causes and risk factors for AKI

An early study of community-acquired AKI admissions into hospital proportionately categorised the underlying causes to pre-renal (hypoperfusion, 70%); intrinsic (10%) and post-renal obstruction (20%). The vast majority of hospital-acquired AKI occurs in “high-risk” patients who are subsequently exposed to multiple sources of injury (Table 1).

1.3.4 Outcomes in AKI

1.3.4.1 Short-term

The most reliable AKI outcome data come from studies of patients admitted to intensive care units or following major surgery. Serum creatinine is linearly associated with an increased risk of adverse outcomes and in studies of patients following cardiac surgery or with heart failure, even small rises predict a poorer prognosis [26]. Patients who develop AKI in hospital have increased
lengths of stay [25] and depending upon co-morbidities, suffer a 10 to 80% risk of mortality [23]. There is reasonable evidence to suggest that AKI is more than a consequence of severe morbidity, but it contributes to other organ dysfunction. Pathways of inflammation and apoptosis within the kidney have been shown to induce maladaptive cellular behaviour in distant organs through metabolic disturbances, circulating activated leucocytes, cytokines and chemokines [27] thus increasing the likelihood of multi-organ failure and associated mortality.

1.3.4.2 Long term

An episode of AKI predisposes an individual to subsequent AKI episodes, established CKD, end-stage renal disease (ESRD) and a higher mortality risk. It seems the relationship between AKI and CKD is bidirectional in that CKD predisposes to an AKI episode whilst AKI can lead to development of CKD. A large systematic review of almost 50,000 AKI survivors reported a CKD incidence rate of 7.8 events per 100 patient-years (although data to present comparative rates in well-matched control patients without AKI were not available) [28]. A retrospective analysis of patients having suffered severe, dialysis-dependent AKI had a 28-fold increased risk of developing CKD stage 4 or 5 over 8-years plus a greater than 2-fold risk of death over hospitalised patient controls without AKI [29]. A study of patients with initial renal recovery (within 30 days) from dialysis-dependent AKI reported a 3-fold higher risk of requiring long-term dialysis over 10 years in comparison to hospitalised age-matched controls who did not receive acute dialysis [30].

Finally, a retrospective study of 3000 patients following cardiothoracic surgery reported worse 10 year survival among patients who had developed AKI following surgery and this was proportional to severity. Even patients with complete renal recovery after AKI had an increased hazard ratio for death of 1.28 when compared to patients without AKI [31].

1.3.5 Treatment of AKI

The treatment of AKI depends upon the underlying cause(s) and it therefore stands that the majority of cases are treated supportively (intra-venous fluids, antibiotics as necessary and avoidance of nephrotoxic agents). Primary
intrinsic renal diseases such as autoimmune glomerulonephritis are relatively rare and dictate their own specific therapies that go beyond the scope of this thesis.

If kidney function declines despite supportive therapy, then renal replacement therapy (RRT; in the form of dialysis or extra-corporeal filtration) may need to be instituted. There are currently no pharmacological agents proven to effectively treat AKI once it is established. Historically, loop diuretics (purportedly to reduce tubular metabolic stress), low-dose dopamine [32] and fenoldopam [33] (increases in urinary flow by reducing sodium reabsorption or through vasodilation, improves renal perfusion) were proposed to be of benefit although none have stood the test of a large randomised control trial to support their routine use [23].

1.4 Chronic Kidney Disease (CKD)

1.4.1 Definition and classification of CKD

Chronic kidney disease (CKD) is broadly defined by the KDIGO group as “abnormalities of kidney structure or function, present for >3 months, with implications for health” [34]. Besides reduced GFR, the most important evidence of damage is persistent proteinuria but can also include persistent non-visible haematuria and structural abnormalities detected by imaging or histology [35].

A classification system proposed by the United States National Kidney Foundation (NKF) based upon eGFR values [36] has been adopted by the UK Renal Association [35] and KDIGO (Table 2) [34].

A normal age-related GFR decline occurs that is independent of any identifiable disease. Longitudinal human study data of creatinine clearances [37] corroborate classic inulin clearance studies in experimental animals [38] that demonstrate decline begins from age 30 onwards at a rate of approximately 1mL/minute/year. It follows that many elderly people will have a GFR in the range of 60mL/min, although often without the other evidence of kidney damage required for a diagnosis of CKD.
<table>
<thead>
<tr>
<th>Stage</th>
<th>GFR mL/min/1.73m²</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>greater than 90</td>
<td>Normal GFR with other evidence of kidney damage (e.g. proteinuria, haematuria or structural abnormalities)</td>
</tr>
<tr>
<td>2</td>
<td>60 to 89</td>
<td>Mild renal impairment with other evidence of kidney damage (e.g. proteinuria, haematuria or structural abnormalities)</td>
</tr>
<tr>
<td>3</td>
<td>30 to 59</td>
<td>Moderate renal impairment</td>
</tr>
<tr>
<td>4</td>
<td>15 to 29</td>
<td>Severe renal impairment</td>
</tr>
<tr>
<td>5</td>
<td>less than 15</td>
<td>Established renal failure or renal replacement therapy</td>
</tr>
</tbody>
</table>

Table 2. K/DOQI guidelines for chronic kidney disease: evaluation, classification, and stratification. Adapted from [36].

1.4.2 Prevalence of CKD

The North American National Health and Nutrition Examination Survey (NHANES) of over 13,000 individuals reported a CKD prevalence of 13% with elevated numbers according to increased age and presence of diabetes or hypertension [39]. Current UK estimates place CKD prevalence between 4 - 8.5% [40, 41]. Given the lack of symptomatology with even moderately reduced renal function, these figures are likely under-representative as a substantial number of cases will not present to medical services and therefore remain undiagnosed.

1.4.3 Causes of CKD

The causes of CKD are diverse, often multifactorial and definitive data on underlying disease is incomplete. Diagnostic histological examination is rarely possible given that most patients present too late in the course of their disease, when a kidney biopsy is often not performed, justified or non-diagnostic (having missed the acute injury).

Population studies and data collections provide some clues as to what conditions underlie the development of CKD although they do not prove a direct causal link. A North American prospective study of almost 25,000 individuals found that diabetes, hypertension and smoking held the strongest associative risk to developing CKD over a twenty-year period [42]. Data from the UK Renal Registry reveals diabetes as the single most common primary renal diagnosis in CKD patients commencing RRT (Table 3).
<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>% patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td>24.2</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>11.6</td>
</tr>
<tr>
<td>Pyelonephritis</td>
<td>7.4</td>
</tr>
<tr>
<td>Hypertension</td>
<td>6.7</td>
</tr>
<tr>
<td>Polycystic kidneys</td>
<td>6.6</td>
</tr>
<tr>
<td>Renal vascular disease</td>
<td>7.5</td>
</tr>
<tr>
<td>Other</td>
<td>16.2</td>
</tr>
<tr>
<td>Uncertain aetiology</td>
<td>19.8</td>
</tr>
</tbody>
</table>

Table 3. Percentage distribution of primary renal diagnosis in a 2010 cohort of patients commencing RRT. Adapted from [43].

1.4.4 Complications of CKD

Although the majority of people with CKD do not develop ESRD, they are at a substantially increased risk of mortality relative to the age-adjusted non-CKD population - largely due to cardiovascular (CV) disease. A retrospective cohort study of over 1000 CKD patients found that only 4% had progressed to RRT within 5.5 years but 69% died - of which, 46% were due to cardiovascular events such as stroke or myocardial infarction [44]. In addition, a recent inter-continental meta-analysis including 100,000 individuals with albumin : creatinine ratio (ACR) measurements and 1.1 million people with urinary dipstick protein measurements found that reduced eGFR and presence of albuminuria were associated with all-cause and cardiovascular mortality independently of each other and other traditional cardiovascular risk factors [45].

The extra-renal complications of CKD begin to manifest from mild CKD (stage 3) and progress with kidney function decline. Patients commonly require exogenous iron and erythropoietin to support physiological haemoglobin levels and chelating agents to temper the effects of rising phosphorus levels and ensuing hyperparathyroidism-related bone disease. In CKD, disordered bone metabolism (termed, CKD-MBD; chronic kidney disease – mineral bone disorder) directly accelerates vascular calcification creating stiff, non-compliant vessels that are narrowed and fail to deliver blood to satisfy metabolic demands. Compounded by a reduction in the oxygen-carrying capacity of the blood (anaemia), the complications of CKD appear to
conspire with one another to reduce overall tissue perfusion and thus enhance morbidity and mortality.

The financial cost implications of CKD mirror the impact of morbidity upon the individual. When costs directly attributable to CKD are combined with the indirect costs (such as RRT, cardiovascular events and hospital stay), total UK annual expenditure is estimated to be £1.45 billion. This figure represents almost 2% of the entire NHS budget and does not take into account spending on complications more discretely related to CKD such as heart failure or bone disease [46].

1.4.5 Progression of CKD

Despite resolution or treatment of the underlying disease, many CKD patients exhibit accelerated rates of GFR decline beyond the expected fall of \( \sim 1 \text{mL/min/year} \) attributed to age. The limited renal specimens taken from patients with progressive renal disease confirm advancing glomerulosclerosis, tubular atrophy and interstitial fibrosis [47] - in keeping with animal models. Furthermore, regardless of the initiating cause, remarkably similar histological patterns are exhibited, prompting the proposition of a “final common pathway”. Further still, the severity of interstitial fibrosis has been shown to reliably predict the development of ESRD [48].

1.4.6 Mechanisms of renal fibrosis

In the aftermath of acute renal injury, inflammation gives way to proliferation and repair although as the prevalence and progression of CKD suggests, there is often failure to completely switch-off these processes. The persistent state of inflammation and fibrosis arises from the interplay between maladaptive renal cell-type responses and ageing, genetic and epigenetic influences. Specific signalling molecules and pathways have been implicated but their relative contribution and interactions are complex and poorly understood (Figure 4) [49].
Figure 4. Factors implicated in renal fibrosis

(A) IFN-γ, Interferon-γ; IL-1, Interleukin-1; MCP-1, Monocyte chemoattractant protein-1; NO, Nitric Oxide; NF-κB, Nuclear factor κB; PDGF, Platelet-derived growth factor; Rho, Ras homolog gene family GTPase; ROCK, Rho-kinase; TGF-β, Transforming growth factor-β; TNF-α, Tumour necrosis factor-α; VEGF, Vascular endothelial growth factor. Adapted from [50].

(B) The interplay of genetics and cellular responses in renal fibrosis. Histological hallmarks include: a persistent inflammatory cell infiltrate with; loss and atrophy of tubular epithelial cells; migration and proliferation of myofibroblasts; matrix deposition and peritubular capillary rarefaction [48].

A popular hypothesis integrates theories of renal injury communication between kidney regions and how this precipitates a final common pathway of worsening fibrosis and functional decline.

1.4.6.1 The final common pathway of CKD

Animal studies performed during the late 20th century underpin the now widely accepted hypothesis that renal function decline is ultimately determined by the number of nephrons and their local glomerular pressures [51, 52]. As nephron number falls due to disease, existing glomerular and tubular structures develop hypertension and hypertrophy in an attempt to maintain the glomerular filtration rate (GFR); so called adaptive hyperfiltration. Over time however, these compensatory changes have deleterious effects. Raised glomerular capillary pressure stretches glomerular walls, enlarging pores that allow passage of plasma proteins. The mechanical strain also stimulates angiotensin II expression which is vasoactive, pro-fibrotic and impairs glomerular sieving function further [53].
The transit of excessive filtrate protein initiates a cascade of pro-fibrotic and proinflammatory signals. Podocytes release pro-fibrotic TGFβ [54] and tubular protein overload induces a tubular epithelial cell stress response to produce reactive oxygen species, cytokines, growth factors and vasoactive molecules that act both locally and downstream (Figure 4A). Inflammatory cell recruitment and activation is triggered and communicated throughout the tubulointerstitium resulting in infiltration of lymphocytes, neutrophils and macrophages.

Interstitial ischaemia is a major factor in renal inflammation and fibrotic progression. Loss of peritubular capillaries is a histological hallmark feature of chronic tubulointerstitial disease and reduced renal parenchyma oxygenation has been demonstrated in animal models of CKD [55]. Hypoxia induces phenotypic change in tubular epithelial cells, fibroblasts and endothelial cells to respectively: drive inflammation, deposit extracellular matrix (fibrosis) and become apoptotic.

Some researchers propose a complete phenotypic transformation of resident tubular epithelial cells into functional myofibroblasts occurs - so called epithelial to mesenchymal cell transition (EMT) [56]). Although this has convincingly been demonstrated in vitro, evidence of such in vivo is lacking. More recently, cellular fate mapping studies identify the pericyte (a myofibroblast precursor that encircles renal capillaries), as the origin of collagen-producing cells seen in fibrosis [57].

Further glomerular destruction [58] occurs due to the inflammatory milieu and extracellular matrix deposition destroys normal architecture, compromising blood flow. The result is a progressive reduction in nephron number and a vicious circle of progressive fibrosis and functional decline is established.

1.4.7 Treatment of CKD

Very few therapeutic agents for CKD are available despite increasing demand from an ageing population and intense basic science and clinical research. The mainstay of CKD therapy currently lies with renin-angiotensin system (RAS) blockade using angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs).
Following landmark studies in CKD patients revealing that proteinuria reduction achieved by intensified blood pressure (BP) control slowed GFR decline [59, 60] a considerable amount of clinical trial activity has been devoted to exploiting this further. Many trials have subsequently demonstrated GFR protection with BP and proteinuria reduction using ACEi therapy over other conventional antihypertensive drug classes such as β-blockers or calcium-channel antagonists [61, 62]. The REIN (Ramipril Efficacy in Nephrology) study was the first to formally isolate the benefit of proteinuria reduction in CKD progression. Despite comparable BP control, ACEi therapy slowed GFR decline and progression to ESRD more effectively than non-ACEi therapy and this effect associated with proteinuria reduction [63]. The proposed mechanisms of ACEi reduction of proteinuria and GFR decline are several-fold. Aside from reducing systemic BP and hyperfiltration, they can boost repair of podocytes, reduce mesangial cell hyperplasia and decrease expression of collagen I/IV and TGFβ [64].

Evidence is now overwhelming for the routine use of ACE inhibitors and ARBs in cardiorenal protection for patients with CKD (those with proteinuric nephropathies perhaps having the most to gain). Alternative mechanisms of CKD progression have been targeted and although drugs exist at various stages of pharmacological development, none are yet proven to provide additional benefit over current approaches (Table 4).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliskiren</td>
<td>Renin blocker</td>
</tr>
<tr>
<td>VDR agonist</td>
<td>Inhibition of renin synthesis</td>
</tr>
<tr>
<td>Pirfenidone</td>
<td>Inhibition of TGFβ</td>
</tr>
<tr>
<td>Bardoxolone methyl*</td>
<td>Antioxidant (NRF-2 agonist)</td>
</tr>
<tr>
<td>Bindarit</td>
<td>Inhibition of MCP-1 (CCL2)</td>
</tr>
<tr>
<td>ET-1 antagonist</td>
<td>Inhibition of ET-1</td>
</tr>
<tr>
<td>Sulodexide</td>
<td>Restores basement membrane</td>
</tr>
</tbody>
</table>

Table 4. Novel drug therapies for CKD under development

Where VDR, vitamin D receptor; TGFβ, transforming growth factor-β; NRF-2, nuclear factor (erythroid-derived 2)-like 2; MCP-1, monocyte chemoattractant protein-1; CCL2, chemokine ligand-2; ET-1, endothelin-1. *withdrawn from phase 3 trial in late 2012. Adapted from [65].

Although CKD appears to progress along a final common pathway, a suitable signalling mechanism to therapeutically exploit is difficult to find. The withdrawal of Bardoxolone from its phase three clinical trial [66] illustrates this
and confirms high specificity is required when targeting complex pathological pathways to protect renal function whilst avoiding intolerable adverse effects.

Nitric oxide (NO) signalling stands as a logical candidate for investigation in renal injury and fibrosis due to clear roles in vascular tone, inflammatory responses and cell signalling. A number of regulatory molecules control NO production and it is their pathophysiology that forms the basis of this thesis.

1.5 The Nitric Oxide Signalling Pathway

Nitric oxide (NO) is a highly reactive molecule present in many different cell types with a diverse range of physiological roles. Its significance emerged in 1987 when the previously described endothelium-derived relaxing factor (EDRF) was identified as NO [67]. NO was shown to regulate vascular tone (arterial smooth muscle relaxation) through activation of soluble guanylate cyclase to catalyse the formation of second messenger cyclic guanosine monophosphate (cGMP) [68]. These initial reports launched a whole new field of biological research that has subsequently implicated NO in a variety of homeostatic systems including neuronal transmission, immune responses, clotting cascades and fibrotic pathways [68].

1.5.1 Nitric Oxide (NO) production

NO is generated from the conversion of L-arginine to L-citrulline by the nitric oxide synthase (NOS) enzymes (Figure 5). Several co-factors are required for NO production; nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH₄) along with the binding of calmodulin protein. Three NOS isoforms exist: nNOS, iNOS and eNOS (respectively: neuronal NOS, NOS-1; inducible NOS, NOS-2; endothelial NOS, NOS-3). As their names suggest, they were first described within specific cell-types or circumstances but have since been identified in a variety of tissue types [69].
The production of nitric oxide (NO)

The NOS catalytic reaction requires the binding of calmodulin protein (not shown) plus the presence of five co-factors: NADPH, nicotinamide-adenine-dinucleotide phosphate; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; BH$_4$, tetrahydrobiopterin; n-, i-, e-NOS, neuronal-, inducible, endothelial-nitric oxide synthase.

The nNOS and eNOS isoforms constitutively generate NO in picomolar concentrations [68]. They are both dependent upon ionic calcium to promote calmodulin binding for activity. nNOS produces NO in nervous tissue for neurotransmission and in the kidney it exerts tonic control over glomerular filtration via expression within the macula densa cells [70].

eNOS is predominantly expressed within vascular endothelial cells, generating paracrine NO to relax vascular smooth muscle cells (VSMCs) thereby regulating tissue perfusion. Vascular eNOS expression increases in response to a number of local signals such as ionic calcium, shear stress and pulsatile flow, while humoral activators include oestrogen and endothelium-depandan vasodilators such as acetylcholine, bradykinin and substance P [69]. Particularly relevant to atherosclerosis, eNOS further protects against vascular injury by inhibiting platelet aggregation, leucocyte adhesion and activation [68].

In contrast to the "constitutive" isoforms, iNOS does not require calcium in order for calmodulin to bind plus it can produce NO in nanomolar concentrations (a thousand times greater than the constitutive isoforms). The expression of iNOS was first described in activated macrophages but has since been reported constitutively in hepatocytes, chondrocytes and renal and pulmonary epithelia [69, 71]. Overwhelming iNOS-derived NO synthesis is well described in inflammatory conditions such as bacterial sepsis. Pathological stimuli such as bacterial lipopolysaccharide (LPS) or
endogenous cytokines [72] induce macrophage iNOS to generate NO in quantities that are cytotoxic to invading cellular pathogens. Although initially advantageous to the host, high concentrations of NO contribute to the vascular hyporeactivity and reduced organ perfusion often observed in septic shock leading to mortality [73]. Animal models of sepsis suggested a benefit from NOS inhibition [74], however disappointingly in human trials, non-selective NOS inhibition increased mortality [75]. This disparity illustrates that global, non-selective NOS inhibition can be detrimental and a more precise target of NO signalling is required to temper excessive NO bioavailability whilst not completely abrogating activity required for homeostasis.

1.5.2 Endogenous regulation of NO

To emphasise the need for intricate NO regulation to maintain homeostasis, biologically relevant regulatory pathways have been described at almost every molecular level; transcription; post-transcription and protein translation; substrate (arginine) and co-factor availability; subcellular localisation, NOS enzyme phosphorylation and neutralisation with nitrogen or oxygen free radicals [76]. A novel mechanism for the regulation of nitric oxide synthesis is via endogenously produced inhibitors of NOS activity: the methylargininines (MAs). These arginine analogues bind to the active site of NOS but inhibit NO synthesis, representing one of very few examples of an enzymatic pathway regulated by endogenous inhibitors [77].

1.5.3 Methylarginine biology

1.5.3.1 Methylarginine production

Methylarginines (MAs) are derived from the methylation of arginine residues within proteins and upon proteolysis, are released as free molecules. The rate of methylation is determined by the protein arginine methyl-transferase enzymes (PRMTs), which produce three MA subtypes; $N^O$, $N^G$-dimethyl-L-arginine (asymmetric dimethylarginine; ADMA); $N^O$, $N^{G'}$-dimethyl-L-arginine (symmetric dimethylarginine; SDMA); and $N^G$-monomethyl-L-arginine (monomethylarginine; L-NMMA) [78]. The PRMT family has to date, 11 isoforms identified by amino acid sequence homology but only 9 of these have demonstrated activity [79]. The PRMTs have been categorised into four
groups according to substrate/product specificity but it is PRMT1 (a Type I PRMT) that accounts for approximately 85% of arginine methylation in vivo, producing both mono- and di-methylations of the same guanidino nitrogen atoms on arginine residues to produce L-NMMA and ADMA [80]. Type II PRMTs (including PRMTs 5 and 7) on the other hand can methylate both guanidine nitrogen atoms which results in symmetric dimethylation to produce SDMA [81].

1.5.3.2 Methylarginine: handling and actions

The biological relevance of MAs became apparent when elevated plasma concentrations of ADMA (8-fold) were reported in patients on haemodialysis compared with healthy controls [77]. In the same publication, administration of ADMA into the forearms of healthy volunteers reduced endothelium-dependent blood flow suggesting inhibition of NOS. Despite numerous subsequent observational studies linking elevated circulating ADMA to CV and renal disease, the extent to which MAs cause or simply reflect disease is yet to be elucidated (discussed further in section 1.6.1).

Although present in human plasma at a 10-fold lower concentration, monomethylated arginine (L-NMMA) can also inhibit NO production but its relevance in disease is uncertain [82]. Plasma SDMA concentrations are similar to those of ADMA (0.38-0.73 μmol/L in health [83], upto 2.42 μmol/L in CKD [84]) but although it does not compete with arginine at the active site of NOS, SDMA can compete with L-arginine uptake into cells in millimolar concentrations in vitro [85]. Until recently, there has been a paucity of evidence to suggest that SDMA is pathogenic in vivo however associations have been reported to exist between serum SDMA and inflammation although it remains unclear as to whether this is NO-independent [86]. Interestingly, a recent study indicated that SDMA may be a chief effector molecule in the transformation of healthy to “unhealthy” high-density lipoprotein (HDL) and subsequent pro-inflammatory toll-like receptor (TLR) activation seen in CKD-related CV disease [87].

Consistent with the presence of endogenous NOS inhibition is the phenomenon described as the “arginine paradox”. Intracellular arginine concentrations far exceed the half-saturating arginine concentration (Km) for
NOS activity although in vivo or in vitro administration of additional arginine increases NO synthesis [88].

MAs liberated during intracellular proteolysis transit between extracellular and intravascular compartments resulting in variable local concentrations. For example, intracellular ADMA concentrations in carotid artery endothelial cells harvested from rabbits were 10-fold higher than plasma levels [89]. Animal studies using radiolabelled methylarginines injected peripherally demonstrated intracellular distribution within an hour – suggesting a constant state of flux between body compartments [90].

Figure 6. Formation, distribution, metabolism and actions of endogenous methylarginines
L-arginine (L-Arg) is present in the circulation at >100 times the concentrations of the free endogenous methylarginines: ADMA and SDMA. ADMA but not SDMA inhibits all 3 isoforms of nitric oxide synthase (NOS), decreasing the production of nitric oxide. L-arginine and the free methylarginines are thought to enter the cell (shown on the left) through the y+ transporter. ADMA and SDMA are generated intracellularly following the methylation, by protein-arginine methyltransferases (PRMT), and subsequent proteolysis, of constituent protein arginine residues. ADMA can regulate protein expression through both NO-dependent and -independent pathways. ADMA but not SDMA is hydrolyzed by DDAH to form dimethylamine (DMA) and L-citrulline (L-Cit), which can be reincorporated into proteins. The majority of ADMA is metabolized by dimethylarginine dimethylaminohydrolase (DDAHs) with the product DMA excreted in the urine whereas SDMA is excreted intact. Both SDMA and ADMA are substrates for alanine-glyoxylate aminotransferase-2 (AGXT2), which is expressed only in kidney (right) and liver (not shown), leading to the formation of symmetrical and asymmetrical α-keto-y-dimethylguanidino valeric acid (DGV) that is also excreted in the urine. Dotted arrows show metabolic pathways for which limited in vivo evidence is available. Acetylation of ADMA has also been described but is not shown. Monomethylarginine (not shown for clarity) is thought to have similar actions, distribution, and degradation pathway to ADMA. Reproduced from [91]

Amino acids are transported across cell membranes via solute carrier proteins, classified according to their tissue distribution and amino acid
selectivity (SLC- prefix). The SLC7 family of y+ cationic amino acid transporters (CATs) transport L-arginine and MAs [92]. MAs can compete with L-arginine at the CAT transporter, representing an indirect mode of NOS inhibition by reducing L-arginine uptake [85].

The intracellular methylarginine : arginine ratio is a critical determinant for NO availability and is dependent upon many factors including protein methylation, proteolysis, cell-trafficking and clearance (Figure 6). Normal plasma ADMA concentrations of <1µM [71] may rise several-fold in renal or CV disease [77] although this does not define what is happening within specific tissues. An appreciation of the time and site specificity of MA handling is required if results of in vivo studies are to be correctly interpreted.

To illustrate this, patients with peripheral vascular disease and increased circulating ADMA were supplemented with dietary L-arginine over a six-month period [93]. Despite a demonstrated increase in L-arginine to ADMA ratio, no benefit in claudication symptoms was found and even a possible reduction in flow-mediated vasodilation was suggested. These data support the notion that plasma L-arginine : MAs concentrations may not reflect those at the site of NOS inhibition and as the authors suggest, counter-regulatory mechanisms may prevent increased NO production in chronic disease states while some suggest an increased amino acid load may even exacerbate vascular disease [94].

Alternative, NO-independent modes of action of MAs have been suggested but remain largely unresolved. There is some evidence to suggest that ADMA directly affects gene expression (particularly PRMT3 and bone morphogenetic proteins [95] and superoxide-mediated apoptotic pathways [96] via NO-dependent and independent mechanisms.

1.5.3.3 Methylarginine clearance

MAs are eliminated in two ways: renal excretion and enzymatic metabolism by methylarginine-metabolising enzymes or MAMEs. MAMEs either hydrolyse MAs; the dimethylarginine dimethylyaminohydroxylases (DDAHS: 1 and 2), or deaminate them; alanine-glyoxylate (AGXT2). The majority (approximately 80%) of free asymmetric methylarginine (LNMMMA and
ADMA) is metabolised by DDAH to produce L-citrulline and dimethylamine (DMA) (Figure 7).

**Figure 7. Endogenous regulation of NO synthesis**

Protein arginine methyl-transferase enzymes (PRMTs) methylate arginine residues within proteins. Arginine and methylarginines are released during proteolysis and become a respective substrate or inhibitor of NO synthase (NOS). The most abundant methylarginine, asymmetric dimethylarginine (ADMA) is hydrolysed by dimethyarginine dimethylaminohydroxylase thus reduced NOS blockade. Both arginine and ADMA are metabolised to L-citrulline. ADMA hydrolysis also produces dimethylamine (DMA).

SDMA on the other hand, is not hydrolysed by DDAH and its clearance is largely reliant upon renal excretion [97]. The second catabolic pathway involving alanine-glyoxylate (AGXT2) has been well described recently [98]. This enzyme is principally expressed in the mitochondria of renal epithelial cells and to a lesser extent in the liver [99]. Although DDAH hydrolysis remains the main metabolic route of methylarginine clearance, AGXT2 has the unique ability to metabolise all three subtypes including SDMA by deamination. Interestingly, genetic deletion of AGXT2 in mice results in raised plasma ADMA and elevated systemic blood pressure suggesting a more significant physiological role for AGXT2 than originally thought [98].

1.5.4 Dimethyarginine dimethyaminohydroxylases (DDAH)

The DDAH enzymes are cytosolic proteins that hydrolyse free ADMA (and LNMMa) to form DMA and L-citrulline. DDAH isoforms 1 and 2 share 63% open reading frame (ORF; the gene sequence for protein coding) homology [100]. Expression patterns of the two isoforms differ markedly and often co-express with specific NOS isoforms. DDAH1 co-localises with nNOS in the
brain and kidney while DDAH2 reflects eNOS expression in the heart, placenta and kidney [101]. Immune cells such as macrophages almost exclusively express the DDAH2 [102]. Further evidence also suggesting a role of DDAH2 in inflammatory responses include; the gene maps to the MHC III region of chromosome 6 (shared by inflammatory mediators TNFα and heat-shock proteins [102]), and the up-regulation of renal tissue DDAH2 protein in a rat streptozocin model of diabetic nephropathy (determined by Western blotting of whole kidney lysates [103]).

1.5.4.1 The biological relevance of DDAH

Both DDAH isoforms are expressed in the vasculature and early studies demonstrate that pharmacological blockade elevates transient ADMA concentrations enough to cause downstream reduction of NO synthesis and vascular contraction [104]. Current technical obstacles to the purification of active recombinant DDAH2 protein make a direct comparison of respective isoform activity impossible however heterozygous deletion of DDAH1 in vivo results in a 45-50% reduction in circulating ADMA, indicating that DDAH1 is the predominantly active isoform [105].

Taken together, these observations indicate that DDAH represents a potential mechanism for in vivo control of NO bioavailability. The highly conserved nature of the protein sequence across species would imply an important biological role. Furthermore, the existence of two isoforms suggests there is some functional specialisation although the nature of this has not been fully explored.

1.6 The DDAH-ADMA-NOS Pathway and Disease

As with any homeostatic system, insufficiency or excess of protein expression or activity can contribute to pathology. Historically, association studies in the field of NO signalling have favoured the notion that elevated ADMA leads to reduced NO bioavailability and is therefore always deleterious. However, emerging evidence from human genetic studies and transgenic models illustrate that too much or too little NO activity has negative pathophysiological consequences and it is the timing and site of dysfunction that is of critical importance.
1.6.1 Disease association studies

A considerable number of reports have implicated dysregulation of the NO-ADMA-DDAH axis in human disease states, particularly in the context of cardiovascular disease and its risk factors (Figure 8). A community population study of 3320 participants in the Framingham Offspring Study identified plasma ADMA as being predictive of death over a 10 year follow up although it was not adequately powered to assess CV disease incidence [106]. Furthermore, a 24 year follow up study of almost 900 healthy women data recognized that a 0.15µmol/L rise in baseline plasma ADMA associated with approximately 30% increase in incident CV events [107]. Numerous other studies report circulating ADMA as a powerful predictor of progressive CV disease and CKD (see Section 1.7.3.2), independent of eGFR and other traditional risk factors [106, 108-111].

Despite this depth of associative data and in most cases, the biological plausibility of elevated circulating ADMA altering NO synthesis, none of these studies prove causality. Given that plasma ADMA rises with CV and renal disease, the potential for confounding remains, even despite adjusting for other risk factors.

Figure 8. Conditions associated with elevated plasma ADMA
CV, cardiovascular; AKI, acute kidney injury [112]; CKD, chronic kidney disease. Adapted from [91, 113].
1.6.2 Human DDAH genetic studies

Population genetic studies provide valuable information on the effects of single gene functional variances on risk and progression of human disease. Functional variants in the DDAH2 promoter region have been associated with improved outcomes in both haemorrhagic stroke and pre-eclampsia [114, 115] and have even shown predictive power for the requirement of vasopressors following cardiac surgery [115].

A loss of function DDAH1 gene promoter insertion polymorphism identified in the Chinese Han population is associated with a reduced DDAH1 mRNA expression in vivo, raised plasma ADMA and significantly increased risk of thrombotic stroke and coronary heart disease [116]. In a small study of 1600 middle-aged Finnish men, a DDAH1 mutant variant with a 1% prevalence and elevation of plasma ADMA, conferred a 5-fold risk of hypertension and a 50-fold risk of coronary heart disease [117].

1.6.3 Animal studies to manipulate NO signalling

The timing and tissue-specificity of NO production clearly impacts upon the balance between advantageous and deleterious consequences. For example, a murine model of pulmonary sepsis showed disease reduction and improved survival with early nNOS and late iNOS pharmacological inhibition however, genetic knock-down of either isoform or timed non-selective inhibition provided no benefit at any time-point [118].

Advances in transgenic technology have produced targeted DDAH gene deletion or overexpressing mouse strains. Together with data from studies using transfection and small-interfering RNA (siRNAs), these animals have provided further insight into the physiological role of DDAH-determined ADMA alterations and their pathophysiological consequences (Table 5).

Despite different transgenic constructs used to delete DDAH1, hetero- or homozygous mice consistently express a hypertensive phenotype with elevated plasma ADMA [105, 119]. Furthermore, endothelial-specific DDAH1 deletion had a significant impact, enough to elevate circulating ADMA and reduce endothelial-dependent relaxation [120]. Meanwhile, transgenic DDAH1 overexpression produces the reverse phenotype, with lower plasma ADMA and reduced systemic BP [121].
Genetic manipulation of DDAH activity has produced mixed effects in disease models. Transgenic or overexpression by transfection of DDAH2 appears to improve function in models of vascular injury [124, 125]. Furthermore, a double transgenic mouse for the proatherogenic apolipoprotein E deficiency and DDAH1 overexpression had lower circulating ADMA and was protected from atherosclerotic plaque formation [126]. In contrast to these findings, DDAH1 overexpression aggravated fibrosis in a bleomycin model of pulmonary fibrosis, while pharmacological DDAH1 inhibition reduced fibrosis and preserved lung function [127].

1.7 NO signalling in the kidney

Many roles of NO within the kidney lead to a common goal: regulation of systemic BP. Although this can only be directly achieved by altering GFR or tubular reabsorption (particularly sodium), the pathways are complex and dynamic, responding rapidly to a host of physiological triggers such as oxygen tension, luminal flow, filtrate composition and neurohumoral stimulation.

1.7.1 Renal expression of NOS and DDAH enzymes

Immunohistochemical and mRNA localisation studies have identified expression and co-localisation of all isoforms of NOS and DDAH in the kidney (Figure 9).
Figure 9. Expression of NOS and methylarginine-metabolising enzymes along the nephron

NOS, nitric oxide synthase; n-, neuronal; i-, inducible; e-, endothelial; DDAH, dimethylarginine dimethylaminohydrolase; AGXT2, alanine-glyoxylate aminotransferase-2.

eNOS is expressed throughout the endothelium of glomeruli and the renal vasculature, proximal tubular cells, thick ascending limb and collecting duct [128], whereas nNOS is expressed in tubular cells of the macula densa [70], principal cells of the collecting duct [129] and pelvic renal nerves. Constitutive expression of iNOS has been identified throughout epithelial regions of the nephron including the proximal tubule, distal convoluted tubule and collecting duct [70].

1.7.1.1 Renal DDAH1 expression in the proximal tubular cell

The expression of DDAH isoforms is equally specific and with respect to DDAH1, it is fundamental to the hypothesis and methods for this thesis.

The proximal tubule is the principal site of DDAH1 expression confirmed by immunohistochemical and micro-Western analysis of micro-dissected segments from the rat [103] (Figure 10). In the same study, DDAH2 protein expression was identified in the glomeruli, macula densa, thick ascending limb, and distal tubule.
Figure 10. DDAH1 protein expression in the normal rat kidney

A and B. Immunostaining showed reactivity in the cytoplasm of proximal tubular cell (bars = 50µm). C. Western blot of microdissected nephron segments; glomerulus (GL); proximal tubule (PT); proximal straight tubule (PST); thick ascending limb (TAL); distal convoluted tubule (DCT); cortical collecting duct (CCD); outer medullary collecting duct (OMCD), and inner medullary collecting duct (IMCD). Reproduced from [103].

Figure 11. Immunohistochemical staining for DDAH1 in normal human kidney

A, anti-DDAH1 antibody showing DDAH1 present in the proximal tubule but not other tubules, glomerulus or vessels. B, anti-DDAH1 antibody pre-absorbed against DDAH1 peptide representing a negative control. Nuclear counter stain with Gill’s haematoxylin. Images provided by Dr Ben Caplin (UCL, unpublished).
Furthermore, immunohistochemical staining of normal human kidney sections within our own laboratory confirms that DDAH1 protein is predominantly expressed in the PT cell (Figure 11). Strong expression of DDAH1 protein has been confirmed in murine whole kidney tissue lysate [105, 120], however cell-type localisation patterns have not been reported in the mouse kidney.

1.7.2 Functional roles of NO in the kidney
1.7.2.1 Renal haemodynamics

The autoregulatory mechanism that protects GFR despite fluctuations in BP is composed of the myogenic and tubuloglomerular feedback (TGF) responses. Arterial infusions of a NOS inhibitor (nitro-L-arginine) in dogs elevated systemic BP and reduced renal blood and urine flow by 25% while markedly increasing renal vascular resistance. These studies reveal that NO maintains the basal low vascular resistance characteristic of the kidney and interestingly, although glomerular autoregulation was blunted, it remained largely intact suggesting that mediators other than NO regulate GFR [130].

1.7.2.2 Tubuloglomerular feedback (TGF)

TGF is a renal homeostatic phenomenon whereby delivery of sodium and chloride ions to the distal tubule is communicated to regulate GFR via the afferent arteriole. The distal tubule of each nephron loops back and positions itself intimately with the vascular pole of the glomerulus – a region called the macula densa. Sodium-potassium-chloride co-transporters (type 2; NKCC2) of the macula densa monitor delivery of chloride [131] and through adenosine or ATP signalling, afferent arteriole smooth muscle cells contract and thus reduce flow. TGF can be studied by measuring GFR or a surrogate such as stop-flow pressure (the PT pressure at which filtration is stopped) during microperfusion. The macula densa has consistently been shown to express high levels of nNOS and the NO produced appears to blunt TGF responses [132]. Microperfusion of individual loops of Henle with all three endogenously-produced methylarginine isoforms (individually) inhibited uptake of co-perfused 14C-labelled arginine via the y+ CAT transporter but only ADMA and LNMMA inhibits NOS in the macula densa. This suggests that in the kidney,
methylarginines not only directly inhibit NOS but can reduce NO production via competitive reabsorption of L-arginine substrate [133].

1.7.2.3 Pressure-natriuresis

Central to the kidney’s control over systemic BP is the phenomenon of pressure-natriuresis. Elevations in arterial BP cause a prompt increase in renal sodium and water excretion (natriuresis and diuresis) in an effort to rid excess volume and return total body salt and BP to normal. Infusion of NOS inhibitor L-NMMA, reduced the pressure-natriuretic response elicited in Wistar-Kyoto rats [134] while a study measuring intra-renal NO by cortically-placed microelectrodes, reported increased NO activity with increments in renal perfusion pressure [135]. This NO was postulated to derive from shear-stress-induced vascular endothelial eNOS up-regulation.

1.7.2.4 Tubular sodium transport

Isolated tubule microperfusion studies allow direct administration of compounds to test effects upon tubular absorption (Jv) of sodium, water and bicarbonate. Administration of the NO-donor, nitroprusside in lower concentrations (10⁻⁶M) stimulated Jv by 50% [136] however at higher doses (10⁻³⁻⁴M), it decreased Jv by up to 70% [136, 137]. Similarly, bimodal effects upon proximal tubular absorption have been observed using the NOS inhibitor, L-NAME, and studies using an nNOS knock-out mouse have produced disparate results [138, 139]. These somewhat frustrating results may well reflect a bimodal response of tubular absorption to NO availability but in addition, are confounded by differences in experimental design, allowing extra-renal mechanisms such as humoral responses and renal innervation to influence results.

The inhibitory effect of NO on individual tubular cell transporters is a little more consistent. Primary tubular cells from rodents treated with NO-donors and cGMP analogues have reduced activity of Na/H, Na/K-ATPase and NHE3 channels compared to controls [140, 141]. Sodium coupled co-transport of glucose, phosphate and amino acids are dependent upon the electro-chemical gradient generated by basolateral Na/K-ATPase. By NO
directly reducing Na/K-ATPase activity, it influences tubular reabsorption of most filtered nutrients.

1.7.2.5 Mitochondrial respiration

Proximal tubular active solute reabsorption is critically dependent upon energy produced in the form of ATP through oxidative phosphorylation within mitochondria. NO is known to influence mitochondrial function at many levels.

Firstly, the vasodilatory effect of NO regulates blood flow and delivery of respiratory substrates and dissipates heat generated by mitochondria [142]. In addition, NO increases blood flow to suit oxygen consumption through the generation of nitrosated haemoglobin (NO binds to oxyhaemoglobin to produce S-nitrohaemoglobin; SNO-Hb) [143]. SNO-Hb donates NO at a rate inverse to the ambient oxygen tension, thereby coupling the regulation of blood flow and oxygen delivery to mitochondria [144].

Interestingly, NO has a bi-directional influence over mitochondrial respiration, determined by chronicity. Acutely, NO reduces oxidative phosphorylation by binding to and inhibiting cytochrome c oxidase – the terminal enzyme in the electron-transport chain [145]. On the other hand, sustained NO exposure has been reported to stimulate mitochondrial biogenesis and increases the mitochondrial DNA content of cells in culture [146]. The same study reports that eNOS -/- mice have reduced mitochondrial mass and both lower basal oxygen consumption and steady state ATP levels.

Finally, the intracellular localisation of NO signalling enzymes (MAME and NOS isoforms) in close proximity to mitochondria supports a regulatory effect upon their function. The non-hydrolysing MAME, AGXT2 has been localised to mitochondria in rodents. There is evidence to suggest a mitochondrial-specific NOS isoform exists, mitochondrial NOS (mtNOS), which is sensitive to ionic calcium [147]. Its proposed location, within the mitochondrial inner membrane, makes it well-placed to inhibit cytochrome c.

1.7.3 NO in kidney disease

1.7.3.1 Acute kidney injury (AKI)

Endothelial NO dysfunction resulting from cardiovascular disease or more acutely, with enhanced iNOS activity during sepsis, can lead to impaired
myogenic autoregulation in the kidney. Failure of the kidney to protect its own perfusion results in the “pre-renal” mechanism of injury frequently seen in clinical practice resulting from sepsis, haemorrhage or following major surgery. Progression of other common forms of renal injury such as drug-related tubulointerstitial nephritis, diabetic renal disease and autoimmune glomerulonephritis are all subject to NO dysregulation (often increased) causing peroxynitrite formation, and inflammatory cell activation leading to chemokine production. The production of NO from iNOS during renal inflammation occurs in glomerular mesangial cells, proximal tubular cells and activated macrophages. The question remains as to whether increased NO bioavailability is chiefly a physiological or pathological response to injury.

Animal studies designed to interrupt NO signalling in acute renal disease have produced disparate results. The administration of NOS inhibitors (L-NIL and L-NAME) intensified cytokine and immunoglobulin responses disease in a rodent model of autoimmune interstitial nephritis [148]. NOS inhibition by depletion of arginine substrate (through administration of arginase) also exacerbated disease in a nephrotoxic nephritis model of glomerular injury [149]. In contrast to these reports, NOS inhibition achieved through restricted dietary L-arginine or L-NMMA administration, reduced acute glomerular injury in an anti-thymocyte model of glomerulonephritis [150]. Results using an iNOS knock-out mouse in a model of glomerulonephritis failed to resolve this controversy. No difference in disease severity was observed, although control animals used in this study were heterozygotes for the transgenic iNOS deletion which restricts the interpretation of these data [151].

1.7.3.2 Chronic kidney disease and fibrosis

While much of the damage relating to AKI inflammation is thought to result from excessive NO activity, the progression of CKD and cardiovascular disease is often attributed to NO deficiency [152]. The kidney is responsible for a large proportion of ADMA clearance through metabolism by DDAH and excretion of the unmetabolised fraction (~20%) in the urine. It is therefore no surprise that plasma ADMA levels rise with declining kidney function although
it is not clear whether this is pathogenic or simply a marker of renal dysfunction.

A number of human population studies correlate plasma ADMA concentrations with CV disease severity and poor outcomes in CKD. One of the largest population studies using data from the Modification of Diet in Renal Disease study, revealed plasma ADMA being not only associated with the presence of CV disease at baseline but a powerful predictor of CV mortality at 10 year follow-up [110].

In patients with IgA nephropathy, higher plasma values of ADMA were associated with severe glomerular and tubulointerstitial damage and a faster rate of eGFR decline. In patients undergoing coronary angiography with CKD stage 3 or 4, plasma ADMA correlated with low GFR, albuminuria and risk of all cause death and CV events [153].

Data from our own group however, questions the notion that raised plasma ADMA is always indicative of ill-health and directly pathogenic. We have previously published work showing a gain-of-function DDAH1 polymorphism that is associated with higher DDAH1 mRNA expression in kidney allografts, lower plasma ADMA levels but a steeper rate of eGFR decline in two independent CKD patient cohorts [154]. These results on the face of it, contradict many association studies that suggest elevated plasma ADMA may not be causal of disease and that more specifically, CKD progression is subject to DDAH1 activity.

Studies using rodent models of CKD report a relative renal NO deficiency. Reduced urinary nitrite and nitrate has been detected in a variety of CKD models of 5/6 nephrectomy, ageing, chronic glomerulonephritis and puromycin aminonucleoside nephrosis (PAN) [155]. Furthermore, interventions to reduce NO availability exacerbated disease in a renal mass reduction model of CKD (single nephrectomy and cautery to the one remaining). In this study, a non-selective NOS inhibitor (L-NAME), an nNOS selective inhibitor (7NI) and genetic eNOS deletion all produced the same deleterious effect [156].

Only a minority of animal studies have attempted to influence CKD progression by manipulation at the level of ADMA-DDAH activity. Direct osmotic pump infusion of ADMA exacerbated renal fibrosis although this
finding was confounded by impressive elevations of systolic blood pressure and were no different from L-NAME infusion suggesting a non-specific hypertensive injury [157]. More convincing are studies in which rodents overexpressing DDAH1 were protected against renal fibrosis and collagen deposition in both angiotensin-induced and nephron-reduction models of CKD [158, 159]. A criticism of these studies however, is their relatively short duration of 4 and 6 weeks respectively, which is arguably too short a period to genuinely reflect the natural history of fibrosis witnessed in human CKD. Furthermore, DDAH1 overexpression was not tissue-specific therefore the positive renal effects may be in part, due to improved systemic BP and vascular reactivity.

1.7.4 The balance of NO: health versus disease

In summary, NO signalling plays a significant role in many homeostatic processes and disease. More specifically, it is implicated in kidney physiological function and responses to acute injury, inflammation and subsequent progressive fibrosis. The proximal tubular cell plays a pivotal role in this pathophysiology and expresses many components of the NO signalling pathway, particularly DDAH1. The overall influence of DDAH upon NO signalling is well understood however the circumstances under which it contributes to renal health and disease remain to be established.
2 HYPOTHESIS

2.1 Hypothesis

“Reduced renal DDAH1 activity protects against kidney function decline”

Objectives

2.1.1 Transgenic model development
To develop and characterise a novel transgenic mouse with conditional DDAH1 deletion in the proximal tubule cell.

2.1.2 Disease model development
To characterise a valid experimental model of kidney disease, manifesting the acute and chronic elements of disease exhibited in humans.

2.1.3 Microperfusion and in vitro studies
(a) Through collaborative isolated tubule microperfusion studies, investigate the impact of ADMA-NO manipulation upon renal tubular function.
(b) To use a proximal tubule cell line and primary culture for in vitro studies to demonstrate a physiological effect of ADMA-NO manipulation and investigate underlying mechanisms of in vivo phenotypes.

2.1.4 A parallel study of renal DDAH2 in Acute Kidney Injury (AKI)
To test the effect of global DDAH2 deletion in an in vivo model of acute renal inflammation.
3 MATERIALS AND METHODS

3.1 In vitro methods

3.1.1 Cell line culture

3.1.1.1 Proximal tubular cell line

Normal Human Proximal Straight Tubular Cells (NHPSTs) were a kind gift from Professor Patricia Wilson. To develop immortalised cells, normal proximal tubular cells (derived from male and female human kidney tissue) in culture were exposed to an immortalising vector, containing pZiptsU19 with the temperature sensitive SV40 T-antigen allele tsA58U19 and a neomycin resistance gene. Neomycin-resistant tubular cells were selected for propagation [160]. For these studies, cells were used from -80°C frozen stock at passage number range 5-9. Initially grown to proliferate at 33°C (permissive temperature for the immortalising T-antigen) they required a further 72 hours at 37°C to switch off the T-antigen before use in experiments. NHPST cells were grown in collagen-coated (50ug/mL Rat tail type I collagen; Beckton Dickinson, one hour incubation and dried overnight) plastic culture plates (Corning) and cells were quiesced by serum-starving for 48 hours prior to treatment. Growth media; 1:1 mix of Click’s medium and RPMI medium (Invitrogen) containing; human transferrin (5ug/mL; Sigma); dexamethasone (50nM; Sigma); insulin (0.5ug/mL; Gibco); 1% fetal calf serum (FCS); penicillin / streptomycin (2mM; Sigma). Cells were fed every 2-3 days and passaged in a 1 to 3 split.

3.1.2 Primary PTC extraction and culture

The development of this technique is discussed in detail in the Results chapter. In brief, mice were sacrificed by cervical dislocation and the renal arterial circulation isolated from proximal branches using suture ligation. Suspended magnetic beads (Invitrogen) were infused via an aortic catheter placed distally to the renal arteries and kidney perfusion confirmed by rapid blanching. Once perfused, both kidneys were dissected out, decapsulated and minced with a scalpel before incubation in a collagenase (Sigma) solution.
at 37°C. The resulting homogenate was successively sieved (100µ and 50µ pore size) and fragments caught by the latter were magnetically separated into glomerular and tubular fractions. Microscopic examination of tubular and glomeruli fractions confirmed purity and magnetic bead entrapment within glomerular capillary loops. Purified tubules were pelleted by centrifugation at 1,000rpm for 5mins before resuspension in media and then plated in uncoated plastic (Corning). Media contained; DMEM/F12 (Invitrogen 21331); 0.5% FCS; 100 iu/mL penicillin; 100ug/mL streptomycin; 0.25ug/mL amphotericin B; 2.2 mM L-glutamine; 10ug/mL insulin or ITS (Sigma I3146 5mL); 5.5ug/mL transferrin; 5ng/mL sodium selenite; 10ng/mL EGF; 5 pg/mL tri-iodothyronine; 5ug/mL dexamethasone. Cells were allowed to settle for 24hrs at 37°C, half volume media change at 48hrs and fed every 2-3 days.

3.1.3 Apoptosis and EMT experiments

Cells were treated with combinations of TGFβ (5ng/mL; Sigma), ADMA (Calbiochem) or DDAH1 inhibitor (L-257; produced by supervisor Dr James Leiper [105]). Experimental groups were harvested from 24 to 96 hours. Western blotting was performed to identify changes in expression of cell phenotype markers and apoptosis. Antibodies to αSMA; E-Cadherin; cleaved Caspase 3; all supplied by Abcam.

3.1.4 Proliferation assays

3.1.4.1 Scratch wound assay

This assay is designed to identify the rate of growth (proliferation and migration) into a deliberate wound scratched across the monolayer of cells in culture. Mitomycin C is a cellular-toxic drug that inhibits cell proliferation allowing the observer to distinguish between that and migration. NHPST cells were grown in 6 well plates as outlined previously. A cross-shaped scratch was drawn across each monolayer using a pipette tip. Cells were washed in PBS prior to incubation with treatment media. Treatment groups included 10% FCS, mitomycin C (2ug/mL, Sigma-Aldrich), DDAH1 inhibitor (L-257; 1,10,100uM), ADMA (1,3,10uM), L-arginine (1mM; Sigma). Cells were photographed over the scratch intersection at time zero, 24 hours and 48 hrs.
Images were quantitatively analysed for percentage growth into the wound using Image J software.

3.1.4.2 Bromodeoxyuridine (BrdU) assay

Bromodeoxyuridine (BrdU) is a nucleoside analogue of thymidine that is incorporated into cellular DNA when dividing in S phase. An indirect antibody system detects incorporated BrdU using a colorimetric plate reader at 450-550nm. Cells were grown in 96 well plates as described previously. BrdU proliferation assay kit (Calbiochem) was used according to the manufacturers’ protocol.

3.1.4.3 MTS assay

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay gives a measure of mitochondrial reductase activity by colorimetry. Mitochondrial activity is taken as a surrogate measure for cell proliferation. Cells were grown in 96 well plates as described previously. MTS assay kit (Promega) used according to the manufacturers’ protocol.

3.1.5 Cell sorting: ImageStream analysis

ImageStream (Amnis, WA, USA) is a cell-sorting system that integrates flow cytometry with fluorescence microscopy - applied in this study to identify YFP-expressing cells in the KAPiCre reporter mouse. Images are taken by automated capture for each cell as they are sorted according to size, shape and fluorescence. Tubular cells were isolated as described previously and subjected to further digestion to obtain a single cell suspension for analysis (Accumax; a protease / DNAse solution; Innovative Cell Technologies Inc, CA, USA).

3.1.6 In vitro metabolism: Seahorse analysis

Real-time aerobic and anaerobic respiration in the NHPST cell line was measured using the Seahorse XF Extracellular Flux Analyzer (Seahorse Bioscience, Copenhagen, Denmark). This automated system quantifies the
cellular respiration rate in basal conditions and when tested using compounds to stress parts of the mitochondrial respiratory chain. Culture plates (24-well) are specially designed to accommodate sensor probes that are lowered into the wells to isolate a 7µL media volume just above the cell monolayer surface. Optical sensors simultaneously measure hydrogen ion production (anaerobic glycolysis) or oxygen disappearance (oxidative phosphorylation) within the confined media volume over several time-points. Compounds can be administered during the assay in order to test maximal respiratory capacity and responses to metabolic stress (Figure 12).

The Seahorse growth plates were collagen-coated and NHPST cells were grown as previously described. NHPST cells were seeded to achieve confluence within 48 hours. After preliminary experiments performed to identify optimal working concentrations for the compounds to be added, cells were treated with ADMA 10 µM, L-257 100 µM and NO donor 100 µM (S-nitroso-N-acetyl-l,l-penicillamine, SNAP) at different time-points; 2, 24 and 48 hours. Protocols were used according to the manufacturer’s guidance notes. Analysis was initially performed using the Seahorse XF24 software, followed by transfer to Microsoft Excel.

![Figure 12. Example bioenergetics profile using the Seahorse Analyser](image)

Compounds are added sequentially to modulate mitochondrial function and their effect on oxygen consumption rate (OCR) is measured. (A) control medium; (B) oligomycin to inhibit ATP synthase; (C) FCCP (carbonyl cyanide 4-[(trifluoromethoxy) phenylhydrazone), an uncoupler to short-circuit the proton circuit and allow maximal respiration; (D) rotenone to inhibit total mitochondrial respiration. Production of lactic acid (hydrogen ions; extracellular acidification rate (ECAR)) can also be measured to quantify anaerobic glycolysis. (Reproduced from Seahorse Analyzer Brochure; www.seahorsebio.com)
3.1.7 Harvesting protein from cell culture

Following treatment, 1.5mL of media was aspirated from each well, centrifuged (14,000 rpm for 15 minutes) and the supernatant stored at -80°C. Cells were washed in PBS, lysed in (RIPA) buffer composed of the following; Tris 50mM; NaCl 150 mM; SDS 0.1 %; sodium deoxycholate 0.5 %; Triton X 100; 1mM PMSF; Complete Protease inhibitor (Roche). Samples were, homogenized using a needle and syringe, centrifuged (14,000 rpm for 15 minutes) and stored at -80°C for future use.

3.1.8 Harvesting RNA from cell culture

At the end of treatment, 1.5mL of media was aspirated from each well, centrifuged (14,000 rpm for 15 minutes) and the supernatant stored at -80°C. Cells were washed in PBS, lysed in Buffer RLT (Qiagen) and stored at -80°C for future use. RNA extraction was performed using column purification kits (Qiagen Ltd, W Sussex).

3.2 Molecular biology methods

3.2.1 Tissue homogenisation

Tissues were lysed by ball-bearing grinding (5mins at 20Hz; TissueLyser II, Qiagen) or a Dounce pestle and mortar homogeniser. Lysates were centrifuged to remove the debris pellet and supernatants stored at -80°C until further analysis.

3.2.2 Tissue protein extraction

Tissue was suspended in PBS containing protease inhibitor (Complete Protease Inhibitor Cocktail Tablets, Roche) and lysed as described above.
3.2.3 Tissue RNA / DNA extraction

Nucleic acids were extracted from animal tissue lysates using column separation kits according to the supplier's protocols (Qiagen RNeasy mini kit for RNA; DNeasy Blood and Tissue kit for DNA).

3.2.4 Protein / RNA quantification

3.2.4.1 Bradford assay

Protein quantification was performed using either a Bradford’s assay or a NanoDrop analyser. The Bradford’s assay is a colorimetric technique for protein quantification. In acidic conditions, Coomassie Blue dye appears red however turns blue as it binds to amino acids. This absorbance shift correlates with protein quantity which can be measured by spectrophotometry [161].

5µL of protein sample was added to 795µL of distilled water and 200µL Bradford dye (BioRad) to a total of 1mL. Controls to generate a standard curve contained bovine serum albumin (BSA) (in serial dilutions of 0; 2; 4; 6; 8; 10; 12 µg/mL) with 5µL of lysis buffer and water made up to 1mL. 100µL of samples and controls was pipetted into a 96 well plate and read by spectrophotometry at 595nm.

3.2.4.2 NanoDrop ™

NanoDrop technology (Thermo Scientific) allows quantification of protein, RNA or DNA using spectrometry at specific wavelengths. It is useful when only small volumes of sample are available as only 1-2uL are required for analysis. Samples (2uL loaded) were measured in triplicates and averaged.

3.2.5 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a molecular biological technique used for amplifying nucleic acids in order to assist sequence analysis. Developed in the early 1980s by American Kary Mullis [162], it earned him the Nobel Prize in Chemistry in 1993. The basic technique has since been adapted to serve a variety of applications including genotyping; quantification of gene transcription; identification of infectious diseases and forensic DNA fingerprinting. In addition to a starting sample of nucleic acid, the components
required for a PCR reaction include complementary DNA fragments or primers (serving as targeted start-points for DNA replication); deoxynucleoside triphosphates (dNTPs) and a heat-stable DNA polymerase. Over a range of temperature increments DNA is denatured into single strands, primers anneal to complementary sequences and DNA polymerase catalyses binding of free dNTPs to form new double-stranded DNA. By repeated subsequent cycles, all new DNA double strands themselves act as templates giving rise to logarithmic amplification. Using this method, samples of nucleic acid can be analysed according to sequence (determined by known primer sequences) and amount (according to rate of replication).

3.2.5.1 End-point polymerase chain reaction (end-point PCR)

End-point PCR assesses the products of PCR after the exponential phase of replication has been reached, at either a pre-defined cycle number or when the DNA polymerase and substrates have been exhausted. For that reason its application is largely qualitative and useful for assessing presence or absence of a sequence (genotyping). Tissue genotyping was performed using a heat-stable DNA polymerase (Taq; a recombinant form of polymerase from the bacterium Thermus aquaticus) to exponentially amplify target regions of DNA. A ready made PCR mix containing Taq polymerase, dNTPs (deoxyribonucleotide triphosphate) and a red dye for loading was used (ReadyMix Taq PCR Reaction Mix, Sigma).

| DNA | 1 µL |
| RedTaq PCR Master Mix (2x), | 6 µL |
| Primers | 0.5 µL each |
| DNase-free water | 4 µL |

**Cycling protocol:**

| Hold | 2 minutes | 94°C |
| 40 Cycles: |  |  |
| 20 seconds | 94°C |
| 40 seconds | 60°C |
| 1 minute | 72°C |
| Final extension | 5 minutes | 72°C |

Table 6. Conditions used for end-point PCR genotyping
A standard PCR protocol is set out above in Table 6. New primers were tested across an annealing temperature gradient (upto 10°C either side of the DNA dissociation temperature; Tm) to find the optimum conditions for polymerisation. Primer sequences are listed in the appendix.

PCR products were separated by agarose gel (1.5%, containing ethidium bromide (EtBr) 0.01%) electrophoresis and EtBr-stained DNA bands were visualised under ultraviolet light (Figure 13).

![Image of PCR products run on agarose gel](image)

**Figure 13. Example image of PCR products run on agarose gel**

Performed for KAPiCre / DDAH1 floxed mouse genotyping. KAPiCre genotyping defines only presence or absence of the gene and cannot distinguish heterozygosity. DDAH1 floxed genotyping reveals an upper band for the floxed allele and a lower band for the WT allele. For instance; H35 is KAPiCre positive and heterozygous for the DDAH1-floxed allele whereas H37 is homozygous.

### 3.2.5.2 Reverse transcription quantitative PCR (RT-qPCR)

Tissue mRNA transcription was determined using a two-step PCR approach first involving reverse transcription to create cDNA from the RNA template followed by quantitative (qPCR) real-time PCR.

Extracted RNA was quantified by nanodrop and 100ng loaded into each PCR reaction using a commercial dNTP/primer/RTase mix (iScript cDNA synthesis kit, BioRad) as outlined in Table 7.
### Table 7. Protocol used for reverse-transcription PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>iScript mix</td>
<td>4 µL</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>1 µL</td>
</tr>
<tr>
<td>RNA 100ng + water</td>
<td>15 µL</td>
</tr>
</tbody>
</table>

**PCR protocol (x1 cycle)**

<table>
<thead>
<tr>
<th>Step</th>
<th>Duration/Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcription</td>
<td>30 min at 42°C</td>
</tr>
<tr>
<td>RTase inactivation</td>
<td>5 min at 85°C</td>
</tr>
</tbody>
</table>

### Table 8. Protocol for quantitative PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sybr Green mix</td>
<td>5 µL</td>
</tr>
<tr>
<td>Primers</td>
<td>0.2 µL</td>
</tr>
<tr>
<td>Water</td>
<td>3.6 µL</td>
</tr>
<tr>
<td>cDNA (1:10)</td>
<td>1 µL</td>
</tr>
</tbody>
</table>

**PCR cycling protocol:**

<table>
<thead>
<tr>
<th>Step</th>
<th>Condition/Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial step</td>
<td>95°C 2min</td>
</tr>
<tr>
<td>Thermal cycling</td>
<td>95°C 3 secs</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C 30 secs</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C 30 secs</td>
</tr>
<tr>
<td>Disassociation</td>
<td></td>
</tr>
</tbody>
</table>

### 3.2.5.3 Quantitative polymerase chain reaction (qPCR)

The 7900HT Fast System (Applied Biosystems) was used for real-time quantitative PCR (RT-qPCR). Sybr Green is a cyanine fluorescent dye that emits light in the green spectrum once incorporated into nucleic acid. A commercial Sybr Green-based PCR mix (iTaQ Fast SYBR Green Supermix with ROX, BioRad) was used according to Table 8.

Samples were amplified in duplicate. Amplification curves were examined for each set and a fluorescence threshold ($C_T$) set manually at the base of the exponential phase of the curve. Amplification efficiency for each primer mix was assessed by running serial dilutions of cDNA (1:4; 1:8; 1:16; 1:32). Standard curve $C_T$ values were plotted on a logarithmic scale and the slope of linear regression used to calculate relative sample sequence transcription. Duplicate $C_T$ values were averaged for each sample and normalised to a housekeeper gene ($\Delta\Delta C_T$ method) to correct for amount and quality of cDNA put into the reaction. Several “housekeeper” sequences were
optimised for different types of biological sample and the most stably expressed gene was selected. Housekeeper genes commonly used included Pol2a, GAPDH, β-actin and α-tubulin. Negative controls were run for each primer set using water instead of template DNA.

3.2.6 Liquid chromatography-mass spectrometry / mass spectrometry (LC-MS/MS)

Liquid chromatography-mass spectrometry (LC-MS/MS) is a sensitive technique for the high-throughput detection of substances down to pico-molar concentrations within complex solutions. The combination of high-pressure liquid-chromatography (HPLC) and sequential mass spectrometry confers heightened specificity.

**HPLC.** Samples in solution are pumped at high pressure down a chromatography column containing adsorbent material such as silica. Solutes bind to the column through hydrophobic and ionic interactions. Elution buffers then wash the column across a pH or salt gradient thus by exploiting differences in column-binding affinity, compounds are separated.

**Mass spectrometry.** Eluted substances pass into the triple quad mass spectrometer where they are first vaporised before ionisation with an electron beam. The ions then travel through an electro-magnetic field (quad or Q1) and separated according to their mass-to-charge ratio (m/z). In the collision cell (Q2) an inert gas such as nitrogen fragments the ions as they are accelerated towards a second m/z ratio-selecting field (Q3) before detection. When the ion and fragment m/z is known for a given compound, the filters can be set to scan specifically for these values. Substances are thus reliably distinguished according to their elution time and subsequent precursor to product m/z ratios (Figure 14).
The Agilent 6400 Series Triple Quadrupole LCMS/MS System was used for this study. Biological compounds optimised for detection include methylarginines (Calbiochem), amino acids; creatinine and lactate (all Sigma).

Biological samples including plasma, urine and tissue lysates were prepared by methanol protein precipitation in a 1:5 dilution, vortexed and the pellet removed by centrifugation. Sample solutions were evaporated to dry in a heat-block and resuspended in mobile phase (0.1% formic acid). An internal standard was added (7-deuterated ADMA; Cambridge Isotope Laboratories, Andover, USA) before precipitation to allow correction for extraction efficiency and ion suppression at the point of detection (ions in large abundance can out-compete minority ions at the detector).

A hypercarb (Thermo Scientific) chromatography column was used and the mobile phase consisted of 0.1% formic acid, 1% acetonitrile (increasing to 50% between 5 and 10 minutes) over a total run time of 15 minutes. The mass spectrum parameters are listed in the appendix. Data was collected using Agilent MassHunter Data Acquisition Software and analysed with MassHunter Qualitative Analysis Software before downloading raw data into MicroSoft Excel for processing.
3.2.7 Urinary proteomics

The urinary peptide profiles of transgenic mice were investigated in a collaborative project with Drs Peter Faull and Pedro Cutillas in The Proteomics Laboratory, MRC Clinical Sciences Centre. The following methodology was written and performed by Peter Faull.

Samples were thawed by removing them from the freezer & placing them into an Eppendorf Thermomixer Comfort mixing instrument set to 22ºC, 900rpm for 10 minutes, and then centrifuged at 5ºC, 13000g for 3 minutes. 200µL of sample was transferred to a 1mL volume Eppendorf Protein LoBind tube where 800µL of 0.1% trifluoroacetic (TFA) acid was added to each to adjust the volume to 1mL. Tubes were mixed at 22ºC, 900rpm for 2 minutes. Samples were then subjected to two different solid phase extraction (SPE) steps. Step 1: Reverse-phase SPE using Waters Oasis HLB cartridges (product code 186000383). Step 2: Cation exchange SPE using Waters Oasis MCX cartridges (product code 186000252). SPE was performed using a manual vacuum manifold (Thermo Scientific, 24 port vacuum manifold) & LC-MS grade solvents from Fisher Chemical.

**Reverse-phase SPE method.** Columns were conditioned with 1.5mL of 100% acetonitrile then equilibrated with two successive 1mL washes of 98% water, 2% acetonitrile, 0.1% TFA. Each 1mL volume of sample was loaded onto individual columns & permitted to bind to the column media. Two successive 1mL washes of 98% water, 2% acetonitrile, 0.1% TFA were added to the column to remove small organic molecules, salts or detergents that could potentially affect ion signal in the mass spectrometer. The media-bound sample was eluted with a single 1mL volume of 40% water, 60% acetonitrile, 0.1% TFA. Cation exchange SPE was performed immediately after collection of eluate.

**Cation exchange SPE method.** Columns were conditioned with 1.5mL of 100% methanol then equilibrated with two successive 1mL washes of 40% water, 60% acetonitrile, 0.1% TFA. Each 1mL volume of sample was loaded onto individual columns & permitted to bind to the media. Two successive 1mL washes of 40% water, 60% acetonitrile, 0.1% TFA was added to the column to remove anionic species that could potentially affect ion signal in the mass spectrometer. The media-bound sample was eluted with a single 1mL
volume of 60% acetonitrile, 35% water, 5% ammonium hydroxide. 1mL sample eluates were transferred to a Thermo Scientific Savant SPD121P SpeedVac Concentrator & dried overnight.

**Sample preparation for mass spectrometry.** Dried samples were solubilised in 40μL of 0.1%TFA, sonicated for 10 minutes and then centrifuged at 5ºC, 13000g for 10 minutes. 10μL of supernatant was transferred to a Bioquote Limited 0.2mL lubricated hydrophobic PCR tube which was placed into an autosampler vial ready for LC-MS injection and analysis.

**Liquid chromatography and mass spectrometry methodology.** A Thermo Scientific (Dionex) Ultimate 3000 nano liquid chromatography system was used to separate peptides prior to mass spectrometric analysis. An injection volume of 4μL was removed from the insert vial and loaded onto a trap column (Thermo Scientific Acclaim Pepmap 100 with dimensions 100μm internal diameter and 2cm length, C18 reverse phase material with 5μm diameter beads and 100Å pore size, product number 164564) at 8μL/min in 98% water, 2% acetonitrile, 0.1% TFA. Peptides were then eluted on-line to an analytical column (Thermo Scientific Acclaim Pepmap RSLC with dimensions 75μm internal diameter and 50cm length, C18 reverse phase material with 2μm diameter beads and 100Å pore size, product number 164540) and separated using a ramped gradient with conditions: initial 5 minutes with 4% B (96% A), then 90 minute gradient 4-55% B, then 10 minute isocratic at 100% B, then 5 minute isocratic at 4% B (solvent A: 98% water, 2% acetonitrile, 0.1% formic acid; solvent B: 20% water, 80% acetonitrile, 0.1% formic acid). Eluted peptides were analysed using Thermo LTQ XL Orbitrap operating in positive polarity, top 4 CID (collision-induced dissociation) method. Ions for dissociation were determined from initial 15000 resolution MS scan (event 1) followed by CID on the top 4 most abundant ions. CID conditions: default charge state 2, 2.0 m/z isolation width, normalised collision energy 35.0, Activation Q value 0.25, activation time 30ms, lock mass value of 445.120030 m/z used.

**Data analysis.** Raw files were searched against UniprotKB/Swiss-Prot database (version 10032012) restricted to mouse entries using Mascot v2.3.01 [163]. Relative quantification of peptides was performed by extracted
ion chromatograms of parent ions using an in-house developed software (PESCAL, [164, 165]).

3.2.8 Biochemical analysis of plasma and urine

Urinary protein measurements were performed using the Bradford’s assay as described above. Urinary glucose was measured with a 96-well plate colorimetric assay using commercially available glucose oxidase reagent (Infinity Glucose (Ox), Thermo Scientific; 50µL of sample plus 200µL reagent, 10min room temperature incubation, read at 490nm). In the reaction, glucose is oxidized by glucose oxidase to gluconic acid and hydrogen peroxide. Hydrogen peroxide reacts in the presence of peroxidase with hydroxy-benzoic acid and 4-amoantipyrine to form a red quinoneimine dye. Colour intensity is proportional to the glucose concentration and quantified with a plate-reader.

Plasma and urinary content of protein, electrolytes and creatinine was performed by automated analysis in the National Health Service laboratory, Hammersmith Hospital.

3.2.9 Western blotting

Proteins were separated and identified using Western Blotting [166] and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels (12%) were made according to the following recipe. Resolving gel (10mL of 12%); 4.35mL dH₂O; 3mL 40% acrylamide/Bis 37.5:1; 2.5mL 1.5M Tris-Hcl pH8.8; 100uL 10% SDS; 5uL TEMED; 50uL 10% APS (all from Biorad). Stacking gel (10mL of 4%); 6.32mL dH₂O; 1mL acrylamide/Bis 37.5:1; 2.52mL 0.5M Tris-Hcl pH6.8; 100uL 10% SDS; 10uL TEMED; 50uL 10% APS. See Table 9 for the detailed protocol. Antibodies for DDAH1 and 2 were developed within our group in conjunction with Sigma Aldrich (goat anti-mouse). GFP antibodies purchased from Abcam and Clontech. Licor secondary antibodies were used for Odyssey fluorescent detection. All other antibodies were purchased from Abcam unless stated otherwise.
Run SDS PAGE
10-20uL protein/well plus 2uL protein marker (All Blue; BioRad). 150V for upto 90mins

Transfer to membrane
Using BioRad Mini-Transblot onto nitrocellulose or Hybond-P (Immobilon), 100V 1 hour, ice cooled

Rinse in PBS Tween 0.1%
10mins

Block in 5% milk (in PBS-T)
Room temperature 1 hour

Primary antibody incubation
4°C overnight in 5% milk PBS-T with agitation, see below for concentrations

PBS-T washes x 6
X3 quick swills, x3 5 min washes with agitation

Secondary antibody incubation
Room temperature 1 hour with agitation

PBS-T washes x 6
X3 quick swills, x3 5 min washes with agitation

Visualisation either:
Chemiluminescence
Amersham ECL kit

Fluorescence detection
Licor Odyssey detection and quantification system

Table 9. Western blotting protocol

3.2.10 DDAH antibody purification

Reliable antibodies specific to DDAH1 and 2 are difficult to produce and outcomes with commercial preparations are variable. Our group successfully generated polyclonal goat antibodies against peptides DDAH1\textsubscript{238-252} and DDAH2\textsubscript{241-255} [167]. It was necessary to purify working solutions of antibody from the sera of peptide-inoculated goats using affinity purification through columns containing beads with covalently bound DDAH peptides. The protocol detailed in Table 10 was adapted from “Using Antibodies – A Laboratory Manual” (Harlow, E. Lane, D [168]). A fraction collector, low-pressure (LP) chromatography system and LP data view software 1.03 were supplied by BioRad.

The eluate was passed through a UV filter set at 280nm to detect protein and allow identification of the antibody-containing fractions that were subsequently run separately through an SDS-PAGE gel and stained with Coomassie Blue to confirm presence of eluted protein. Antibodies were tested against liver and kidney lysates from WT animals and global DDAH1 and DDAH2 knock-out animals for negative controls. As expected from non-homologous peptide sequences to which they were raised – DDAH1 and 2
antibodies exhibited no cross-reactivity and in addition, observed band sizes were different; DDAH1 ~37kDa, DDAH2 ~28kDa.

<table>
<thead>
<tr>
<th>Column washing</th>
<th>Sequential 20mL volumes at 2mL/min: 10mM Tris (pH=7.5); 100mM glycine (pH=2.5); 10mM Tris (pH=8.8); 100mM triethylamine (pH=11.5); 10mM Tris (pH=7.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>20mL goat serum in 20mL Tris (pH=7.5), spun 5min, 4000rpm. Supernatant ran at 0.5mL/min</td>
</tr>
<tr>
<td>Washing column (to clear non-specific bound protein)</td>
<td>40mL volumes at 2mL/min: 10mM Tris (pH=7.5); 500mM NaCl/Tris (pH=7.5)</td>
</tr>
<tr>
<td>Acid elution (of bound antibody)</td>
<td>20mL of glycine (pH=2.5) at 1mL/min. Fractions collected into tubes containing 200uL Tris (pH=8.8) to immediately neutralize acid</td>
</tr>
<tr>
<td>Washing</td>
<td>20mL of Tris (pH=8)</td>
</tr>
<tr>
<td>Base elution</td>
<td>20mL of triethylamine (pH=11.5)</td>
</tr>
<tr>
<td>Washing</td>
<td>40mL of Tris (pH=7.5)</td>
</tr>
</tbody>
</table>

Table 10. Detailed protocol for DDAH antibody affinity purification

3.3 Biochemical methods

3.3.1 Nitric oxide quantification

As previously described NO is highly reactive, making direct measurement in ex vivo samples impractical. Assays are therefore designed to detect levels of stable end products of NO metabolism; nitrite (NO$_2^-$) and nitrate (NO$_3^-$). Assessments of NO activity in vivo using nitrite and nitrate species (NOx) as surrogates is an accepted approach, however a significant effect of dietary nitrate has to be considered. Ideally, in vivo experiments using NOx as an end-point measure should be performed whilst subjects are fed a low nitrite/nitrate diet to reduce variability and better expose genuine effects of intervention [169, 170]. In these studies, a low nitrite/nitrate diet was not used and therefore results have to be interpreted with this as a consideration.
3.3.2 Nitric Oxide Analyser

The Sievers NOA 280i (GE Analytical Instruments, Colorado, USA) was used to measure the NO content of small volumes of biological samples. Nitric oxide is re-derived from nitrites and nitrates (stable end-products of NO activity) by reduction in heated vanadium chloride. NO is detected and quantified in a gas-phase chemi-luminescent reaction with ozone which emits in the red/infra-red spectrum.

The lower limit of detection is around 0.2µM for a volume of only 5µL. Samples prepared by methanol protein precipitation (1:5 volume mix; vortex; high speed 10min centrifuge to remove pellet), run in triplicate volumes of 20µL, averaged and NO quantified by calculation against a standard curve of sodium nitrate (0-200µM). Data was recorded using Sievers NOAnalysis Liquid software.

3.3.3 Griess reaction

This method quantifies organic nitrite and utilizes the chemical diazotization reaction originally described by Peter Griess in 1879. Sulfanilamide forms a diazonium salt with organic nitrites and subsequently N-1-napthylethylenediamine dihydrochloride (NED) reacts under acidic conditions (phosphoric acid) to form a pink colour, which can be read by colorimetry at 540nm.

The Griess assay is less sensitive than chemi-luminescence with a lower limit of detection of approximately 1µM. In addition, at least ten-times the volume of sample is required (50µL) however the assay is fast, inexpensive and allows high numbers of samples (in 96 well plates) to be tested simultaneously. 1% (w/V) sulphanilamide / 5% phosphoric acid is mixed in a 1:1 ratio with; 0.1% (w/v) N-(1-napthyl) ethylenediamine and added to an equal volume of sample (50-100µL/well). A standard curve of known sodium nitrite concentrations (0-100µM) was used for quantification. (All chemicals purchased from Sigma).
3.4 Histological methods

3.4.1 Frozen section histology

Tissue harvested from the ROSAYFP reporter mouse was fixed overnight in 4% PFA and then 30% sucrose in PBS until the tissue sank (around 4 hours). Specimens were then embedded in OCT and snap frozen onto cork discs in either isopentane cooled in liquid nitrogen or methanol cooled in dry ice and stored at -80°C.

For frozen sectioning samples were warmed to -20°C in the cryotome (CM 1850 Leica Microsystems, Germany) before cutting 5 µm sections onto poly-L-lysine-coated glass slides. Sections were dried at room temperature and coverslips mounted using a reagent containing DAPI for nuclear staining (Prolong® Gold, Life Technologies™). Slides were studied using a Leica SP5 microscope.

3.4.2 Immunohistochemistry

3.4.2.1 Tissue preparation

Harvested organs were fixed in 4% paraformaldehyde (PFA) overnight and then stored in 70% ethanol. For kidney specimens, half of the right kidney was transected in the sagittal plane. Samples were processed and stained by staff at the Department of Histopathology, Imperial College at South Kensington, London, unless stated otherwise. Tissues were embedded in paraffin blocks by an automated processor and 5µM sections cut on a microtome and attached to poly-L-lysine-coated glass slides.

3.4.2.2 DDAH1 staining

The detailed protocol in Table 11 was used for DDAH1 staining. Initial staining for DDAH1 in mouse and human kidney was performed upon sections previously prepared and kindly donated by Dr Ben Caplin (UCL, London).

3.4.2.3 Periodic Acid Schiff (PAS) Stain

Periodic acid reacts with carbohydrate molecules to create aldehydes giving a purple colour upon reaction with Schiff reagent. PAS staining is therefore
useful for the histological assessment of carbohydrate-rich structures such as the tubular and glomerular basement membranes in kidney sections.

Sections were rehydrated through xylene and graded ethanol (100%, 95%, 70%) solutions to water. Following rehydration, slides were submerged in periodic acid for 5 minutes, washed and then exposed to Schiff’s reagent for 1 hour. Following further washing, sections were counter-stained with Gill’s haematoxylin for 3 minutes, soaked in tap water for 5 minutes and destained in acid alcohol. Slides were dehydrated in xylene and coverslips applied using DPX mountant.

<table>
<thead>
<tr>
<th>Wax removal</th>
<th>10 min xylene soak, then xylene washes x2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100% ethanol washes x3</td>
</tr>
<tr>
<td></td>
<td>5 min water soak</td>
</tr>
<tr>
<td></td>
<td>10 min soak in methanol plus 6% hydrogen peroxide</td>
</tr>
<tr>
<td>Antigen retrieval</td>
<td>Citrate buffer (0.021%) 5 min then 20mins on “HIGH” in microwave</td>
</tr>
<tr>
<td></td>
<td>Water wash</td>
</tr>
<tr>
<td>Blocking</td>
<td>5 min PBS soak</td>
</tr>
<tr>
<td></td>
<td>20 min normal horse serum incubation (3 drops; Vector)</td>
</tr>
<tr>
<td></td>
<td>PBS wash</td>
</tr>
<tr>
<td></td>
<td>15 min Avadin (3 drops; Vector), PBS wash</td>
</tr>
<tr>
<td></td>
<td>15 min Biotin (3 drops; Vector), PBS wash</td>
</tr>
<tr>
<td>Primary antibody</td>
<td>DDAH1 goat polyclonal (1:50; Dr J Leiper), 1 hour at room temperature</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>Biotinylated horse anti-goat IgG (1:200; Vector), 30 min room temperature. PBS wash</td>
</tr>
<tr>
<td>Tertiary antibody</td>
<td>ALP ABC kit (1:100; Vector), 30 min room temperature. PBS wash</td>
</tr>
<tr>
<td>Substrate staining</td>
<td>ALP substrate kit (Vector), upto 30 min room temperature, stopped by water wash when staining clear</td>
</tr>
<tr>
<td>Counter-stain</td>
<td>Mayer’s haematoxylin 5 min</td>
</tr>
<tr>
<td>Washing</td>
<td>x 3 sequential washes in ethanol and xylene</td>
</tr>
<tr>
<td>Mounting</td>
<td>DPX and coverslip, dry overnight</td>
</tr>
</tbody>
</table>

**Table 11. Protocol for DDAH1 immunohistochemical staining**
3.4.2.4 Picrosirius Red Stain

Following rehydration, slides were stained with picrosirius red (Direct Red 80 0.5% in saturated picric acid) for 1 hour. Slides were then washed and counter-stained with Weigert's haematoxylin (an acid-resistant nuclear stain) for 2 hours before washing, dehydration and coverslipping.

Although collagen fibre deposition is frequently demonstrated with picrosirius red staining, there is a lack of consensus as to the best method of visualisation [171]. Bright field visualisation allows detection of red-stained collagen but lacks specificity and although linearly-polarised light improves upon this, a proportion of fibres lying parallel to the transmission axis will inevitably be invisible. Circular polarised light removes this problem and allows all collagen fibres to be visualised.

The sections were analyzed using a Manual Zeiss Axiophot microscope (Zeiss, NY, USA) equipped with filters to provide circularly polarized illumination. The lower filter (3M circular polarizer; Edmund Industrial Optics, Barrington, NJ, USA) was placed above the microscope’s field iris diaphragm ring, while the upper filter was constructed from a combination of a quarter-wave plate (U-TP137, Olympus) placed below a linear polarizer aligned such that its transmission axis was at 45° to the fast axis of the wave plate. These two filters were aligned and fixed to maintain a field background as dark as possible (that is, the filters were “crossed”). Tissue images were obtained with a 2.5X objective lens, recorded on a digital camera (Canon EOS-40D digital SLR; Canon Inc, Tokyo, Japan) and viewed through Canon Utilities EOS software.

Images were subsequently analysed using a Fiji software macro written by Dr Dirk Dorman, Head of Microscopy, MRC Clinical Sciences Centre, Imperial College, London, which automated the following steps to quantify collagen in each whole kidney slice: individual images of each section were stitched together to form a whole kidney image; a plane slide image was used to correct for background; whole kidney area in pixels was recorded using texture thresholding; whole collagen area (yellow-green birefringence) was recorded by colour intensity thresholding. Relative collagen deposition per total section area (pixels) could then be reported. Using the whole kidney
section and automated analysis removes potential bias and therefore the need for observer blinding.

3.5  *In vivo* methods

3.5.1 Regulatory Approval

Animal experimentation was undertaken only in accordance with the 3R principles of replacement, refinement and reduction. *In vivo* procedures were used only where no appropriate *in vitro* methodology was available and experiments were designed to minimise suffering to the animals involved. All animal procedures were conducted under the terms of Home Office Project and Personal Licences.

Where possible, power calculations were used to guide what minimum number of animals would be adequate for experiments. An online power / sample size calculator was used (http://www.stat.ubc.ca/~rollin/stats/ssize). As an example, using the relative percentages of DDAH1 protein expression between tubular and glomerular lysates; a power of 80% was deemed adequate to detect a minimum 25% difference between the two groups. An estimated standard deviation size (σ) of 15% was derived from values gained in previous Western blotting assays using the DDAH1 antibody. Statistical significance (α) was set at 0.05 for a two-sided test.

3.5.2 Housing Conditions

Animals were maintained in social groups where possible with appropriate environmental enrichment (e.g. bedding materials, mouse tunnels). Animals were kept in rooms at a constant temperature and maintained on a 12-hour light/dark cycle. They were fed on standard rodent chow and water *ad libitum* unless stated otherwise.

3.5.3 Experimental animal strains

The transgenic constructs and breeding strategies are described in further detail in Chapter 4: “Targeted gene deletion in the renal proximal tubule”.
3.5.3.1 The floxed DDAH1 mouse

The DDAH1 floxed mouse strain was bred on a mixed B6/129 background. The construct was developed in-house by our own group, wherein LoxP sites were inserted to flank exon 1 of DDAH1, which encodes the initiating methionine residue and the first 100 amino acids of the protein (see Chapter 4.4).

3.5.3.2 The KAPiCre mouse

The KAPiCre transgenic mouse developed by Li et al [172] was purchased as a male heterozygote on a C57BL/6J background from Jackson Laboratories (strain name: B6.Cg-Tg(Kap-cre)29066/2Sig/J). (See Chapter 4.2.1).

3.5.3.3 The ROSAYFP mouse

The ROSAYFP (heterozygote male) mouse on a mixed B6/129 background was a kind gift from Professor Graham Williams. (See Chapter 4.3).

3.5.3.4 The DDAH2 global knock-out mouse

The DDAH2 global knock-out transgenic mouse line was created through the Texas Institute for Genetic Medicine (TIGM, Houston, Texas, USA) and bred on a mixed B6/129 background. DDAH2 gene disruption was achieved by the insertion of a long terminal repeat into the first exon of the gene using a retroviral vector (http://www.tigm.org/). (See Chapter 11).

3.5.4 Transgenic Animal Genotyping

At approximately 4 weeks of age, mice were genotyped using tail-clipped tissue and ear-notched for identification. DNA extraction was achieved using “GNK” lysis buffer (Triz base 10mM; MgCl₂ 1.5mM; KCl 50mM; gelatin 0.01%; Igepal Ca-630 0.45%; Tween 20 0.45%) with fungal proteinase K. Samples were digested overnight at 53 °C and the proteinase K deactivated by a 10 minute period at 100 °C. End-point PCR using 1µL of DNA per reaction mix was performed as described previously. Primer sequences for transgenic genotyping were obtained from Dr J Leiper (DDAH1 floxed, DDAH2 KO) and the Jackson Laboratory (KAPICre) (see appendix).
3.5.5 Urine Collection

Animals were placed in metabolic cages for a maximum of 24 hours. Collected urine was centrifuged at high speed for 10 minutes, the pellet discarded and stored at -80°C.

3.5.6 Testosterone pellet insertion

Exogenous testosterone was administered in the form of a pellet placed subcutaneously. The original investigators used pellets containing 5mg of testosterone designed to release over 21 days, equivalent to 0.24mg/day \[172\]. To cover a 12 week CKD model, testosterone pellets for this study were made to request for a similar daily dosage of 0.24mg/day; 22mg continuous release over 90 days (13 weeks; supplied by Innovative Research of America, Florida, USA). Mice were anaesthetised under oxygen (2L/min) delivered isoflurane and placed on a warming mat. After shaving, a small 3-5mm incision was made in the nape of the neck to the right of midline. A subcutaneous pocket was created by blunt forceps dissection, the pellet inserted and the incision closed with two sutures using 5-0 Mersilk (Ethicon, Johnson and Johnson Medical Ltd. UK). Animals were allowed to recover in a heated chamber (32°C) and checked daily for two days following surgery.

3.5.7 Salt-feeding

In some experiments, selected mice were given low- or high-sodium chow (0.03% or 3.15%; TestDiet®, IN, USA) with ad libitum access to normal water. Urine was collected in metabolic cages before and after 8 days of salt-feeding before blood pressures were recorded under terminal anaesthesia. Plasma and kidney tissue was harvested as described elsewhere.

3.5.8 Models of renal injury

3.5.8.1 Folate model

The use of intraperitoneal (IP) folate (folic acid or pteroylglutamic acid) to model renal injury is a well-established experimental technique \[173, 174\]. Folate induces a dose-dependent nephrotoxicity as crystals precipitate within renal tubule lumina. Acute tubular necrosis ensues within 48 hours, followed
by a period of epithelial regeneration and from 8 weeks following injury, kidneys exhibit loss of function and by 12 weeks, progressive fibrosis (Figure 15).

![Progressive fibrosis in experimental mouse folate nephropathy](image)

**Figure 15.** Progressive fibrosis in experimental mouse folate nephropathy
Female mice on a CD1 background received a single IP folate dose of 200mg/kg at T0. Assessment of fibrosis performed by semi-quantitative analysis. Reproduced from [175].

Folate injury thus provides a reproducible model with which to investigate the natural history of inflammatory pathways from initial acute tubular injury through to chronic kidney disease. Time points of 2, 14 and 84 days were chosen for this study to permit assessment of the acute injury phase, recovery and eventual progression to chronic fibrosis respectively.

Mice received a single intra-peritoneal injection of folate (240µg/g of body weight) dissolved in 0.3M NaHCO₃ vehicle (20mg/mL) or vehicle control alone (both from Sigma). Mice were sacrificed by CO₂ narcosis followed by cervical dislocation at day 2, day 14 or day 84 (12 weeks). Blood was collected by direct cardiac puncture and stored on ice before high-speed centrifugation to remove the plasma. The right kidney was halved (sagittal plane) for histology, and quartered for future RNA and protein extraction while the left kidney was weighed. Samples were stored at -80°C prior to further processing. Prior to sacrifice, urine was collected in metabolic cages for the 14 and 84 day time-points.
3.5.8.2 Nephrotoxic nephritis model

Nephrotoxic globulin and expertise with this model was kindly provided by Dr Ruth Tarzi. The following is a description of how this was prepared [176]. Sheep were immunized with murine glomerular lysate initially in complete and then incomplete Freund’s adjuvant (Sigma, Dorset, UK). Ammonium sulfate precipitate was dialyzed against sterile PBS (Invitrogen, Paisley, UK) and stored at -80°C. (The endotoxin content of the nephrotoxic globulin was undetectable after using a Biowhittaker QCL-1000 LPS assay kit (Biowhittaker, Walkersville, MD).

Mice were pre-immunized with intra-peritoneal sheep IgG (0.2 mg) in a 1:1 volume mix with complete Freund’s adjuvant (Sigma). Five days after the immunization, 5 mg of nephrotoxic serum (NTS) was injected through a tail vein. The mice were monitored clinically; 10 days after NTS injection, the mice were sacrificed and harvested as described above.

3.5.9 Invasive Blood Pressure Measurement

Invasive blood pressure recordings were performed with the assistance of Dr Zhen Wang. Animals were anaesthetised under oxygen (2L/min) delivered isoflurane anaesthesia and placed on a warming mat. The carotid artery was isolated and a ‘Mikro-Tip’ pressure transducer catheter (Millar Instruments, TX) was introduced. Recordings were obtained using the Powerlab system and data recorded and analysed using Chart software (both from ADInstruments, Oxfordshire). Isoflurane anaesthesia was minimised to 1% and after a 3-minute stabilisation interval, blood pressure was recorded and sampled for approximately 50 cardiac cycles. All measurements were blinded to genotype and performed between 0800 and 1300 to maintain diurnal consistency.

3.5.10 Isolated proximal tubule microperfusion

These studies were done in collaboration with Professor Chris Wilcox’s group at the Kidney and Vascular Research Center, Georgetown University, Washington, USA. Dr William Welch and Professor Wilcox supervised the work whilst Miss Tracy Bell provided the surgical expertise. This technique measures intrinsic tubular epithelial transport in vivo and historically, data
gathered in this way forms the basis of renal tubular physiology. Single proximal tubule (PT) segments are isolated by cannulation and by administering various compounds, the roles of key regulators of PT function can be tested. The technique permits control of flow rate, sodium concentration, osmolality, and extracellular pH. The methods, performed in both rats and mice, have been described previously [177, 178] but a brief outline follows.

3.5.10.1 Animal preparation

Animals were anesthetized with thiobarbital (Inactin, 80 g/kg IP; Research Biochemicals Inc) and cannulae were placed in a jugular vein for infusion of fluids and in a femoral artery for the recording of mean arterial pressure (MAP; Powerlab, AD Instruments Inc). A tracheotomy tube was inserted and animals ventilated room air spontaneously. A catheter was inserted in the bladder and another in the left ureter to collect urine. The left kidney was exposed by a flank incision and stabilized in a Lucite cup (Vestavia) mounted on a heated surgical table and bathed in mineral oil maintained at 37°C (Figure 16).

Figure 16. Renal proximal tubule micro-perfusion in rodents

A. The animal is anaesthetized on a heat mat and cannulated as labeled. B. The left kidney is held in a cup and bathed in mineral oil heated to body temperature. C. A glass micropipette is advanced into a superficial tubule under microscopic guidance and optic light source (dye is visible in the tip which is used to confirm successful cannulation).
After surgical preparation, rats were infused with a solution of 154 mmol/L of NaCl and 1% albumin at 1.5 mL/h to maintain euvoemla. Studies began after a 60-minute of stabilization period.

3.5.10.2 Microperfusion of PTs

The kidney surface was examined microscopically and a PT segment of appropriate visible length was injected with a “finding” pipette containing dye-stained artificial tubular fluid. The flow was blocked by injection of T grease (T grade, Apiezon Products) via a micropipette (10 to 12 µm OD) proximal to the perfusion site. The tubule was perfused with a micropipette (8 to 10 µm OD) connected to a micro-perfusion pump (model A1400, World Precision Instruments Inc) at 18±3 nL/min. The perfusion solution contained 14C inulin (New England Nuclear) as volume marker and 0.1% FD&C green dye for identification of the perfused loops.

Tubules were perfused for 2 to 8 minutes before fluid collections, which were made at a downstream site with a micropipette (7 to 10 µm OD) after placement of a column of oil to block downstream flow. Samples were collected for 3 to 5 minutes and transferred into a constant-bore capillary tube for which the length was measured with a micrometer to calculate the tubular fluid volume. Thereafter, the samples were injected into scintillation fluid and the 14C activity counted. Collected samples with <95% and >105% of microperfused inulin were discarded. The amount of microperfused inulin was estimated by the average of 14C-activity in 4 samples microperfused directly into capillary bore tubes at the end of the experiment. To determine the lengths of the perfused segments, tubules were filled with high-viscosity microfil (Flow Tech, Inc).

At the end of the experiments, the kidney was partially digested in 20% NaOH, and the casts were measured under a dissecting microscope. The Jv was calculated by the difference in the perfusion rate and the collection rate factored by the length of the nephron: Jv = Vperf (nL/min) - Vcoll (nL/min) / PT length (mm) where Vperf indicates perfusion rate and Vcoll indicates collection rate and expressed as nL/min/mm. The composition of the perfusion fluid was as follows (mM): 125 NaCl, 20 NaHCO3, 5 KCl, 1 MgSO4, 2 CaCl, 1 NaHPO4, 5 glucose, and 4 urea.
5.10 Data analysis
Statistical analysis was performed using the Prism software package (Graph Pad Inc., UK). All data are presented as means ± SEM. Distribution of data was determined by skewness and D’Agostino and Pearson omnibus normality test for group sizes of more than 7 (below which normality testing is inaccurate). For comparisons between two groups for a single variable, an unpaired two-tailed t-test (parametric data) or a Mann-Whitney U test (non-parametric data or where a normal distribution could not be assumed) was used. Differences between multiple groups were assessed by one-way analysis of variance (ANOVA) followed by Bonferroni’s post test for multiple comparisons. Two-way ANOVA was used where comparisons were being made between groups subject to two experimental variables, followed by Bonferroni’s post test for multiple comparisons. P values of <0.05 were considered statistically significant.
4 Targeted Gene Deletion in the Renal Proximal Tubule

4.1 DDAH1 expression within the proximal tubule

As discussed in Section 1.7.1.1, the proximal tubule has been demonstrated to be the principal cell type to express DDAH1 protein in both the rat and human kidney. Further immunohistochemical staining of human kidney tissue for this study reproduced a similar pattern of expression (Figure 17).

![Figure 17. Immunohistochemical staining for DDAH1 in normal human kidney. A. DDAH1 staining is seen in the epithelial cells of the proximal tubule (arrows) and not in the glomerulus (g) or control (B; DDAH1 antibody pre-incubated with DDAH1 peptide).]

In the mouse, qPCR and Western blotting analysis of extracted tubular cells indicated that DDAH1 transcription and protein expression is approximately 95% higher in kidney tubule lysates when compared with glomerular lysates (Figure 18).

![Figure 18. DDAH1 expression in murine tubular cells. A. DDAH isoform protein expression and B. MAME transcription. DDAH1 protein expression predominates in tubular fractions. Consistent with previous reports, DDAH2 expression is stronger in the glomeruli, and AGXT2 in the tubules. Comparisons are made between tubular (white bars) and glomerular fractions (black bars) of WT mouse kidney tissue (corrected relative percentages). Mann-Whitney U test, n=5, *** p<0.001.]

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4.2 Transgenic technology

Genetic modification or transgenic technology developed over the last two decades has revolutionised the study of the roles specific genes play within whole organisms. Transgenic techniques are most well developed in the mouse, owing to advanced knowledge of its genetics, ability to manipulate embryonic cells (ESs), rapid breeding times and relative small size with low maintenance costs. Two basic approaches are used to either overexpress or delete a gene; random chromosomal integration and homologous recombination.

4.2.1 Random chromosomal integration

DNA sequences (a promoter element and gene of interest) are microinjected into a fertilised oocyte that is subsequently inserted into a pseudopregnant female. This technique is most often used to achieve de novo or exaggerated expression of a gene whose product may either be functional or dominantly inactivating against a native protein. In addition, this technique can be used to introduce a “reporter” protein, such as green fluorescent protein (GFP), that is functionally innocuous but expression is driven by upstream regulatory elements. By using immunohistochemistry or biochemical assays to determine the expression pattern of GFP, insights can be gained into the presence and functional role of upstream control sequences. Similar random DNA integration can also be achieved using a viral vector to transflect the target cells in order to express a new gene product.

Interpreting the phenotypes of animals created in this way requires caution. New DNA insertion is random and can occur within inactive sites of the chromosome or interrupt native gene sequences. Furthermore, a transfected cell overexpressing protein in supra-physiological concentrations may undergo adaptive changes to the expression and function of other genes resulting in a phenotype not necessarily reflective of the function of the gene under scrutiny.

4.2.2 Homologous recombination

Homologous recombination is a normal biological phenomenon of nucleotide sequence exchange between similar DNA strands that repairs harmful breaks
and produces small genetic variations. The process can be exploited for the targeted introduction of new gene sequences using a technique developed by Capecchi, Evans and Smithies for which they won the 2007 Nobel Prize in Physiology or Medicine [179].

A specifically designed DNA construct flanked by sequences identical to the targeted locus is introduced into embryonic stem (ES) cells. After translocation to the nucleus, the construct replaces the targeted locus through homologous recombination. ES cells containing the recombined DNA are then injected into developing embryos and if integration is successful, the developing fetus is chimeric: composed of two different cell populations – some containing all native DNA and others, the new DNA construct. If the fertilised oocyte is from the transgenic sub-population, subsequent breeding will produce heterozygotes for the new genotype, allowing generation of homozygotes from successive mating. This technique is commonly used to delete or disrupt selected genes to create a null or “knock-out” animal. The approach also allows for the study of gene expression “dose” effects, by comparing the phenotypes of wild-type and transgenic homozygous organisms against those with haploinsufficiency.

A number of limitations exist with homologous recombination. Many genes are crucial for embryonic development and their deletion results in lethality. Even in viable offspring, absence of a gene throughout development to adulthood can provoke adoptions to the expression of other genes that lead to a phenotype not necessarily representative of the deleted sequence. In addition, non-specific tissue type gene deletion makes phenotype interpretation difficult. For example, global DDAH1 deletion increases circulating ADMA, elevates systemic BP and may influence renal autoregulation - introducing strong confounding variables when trying to define the specific pathophysiological roles of renal proximal tubular DDAH1 activity. Cre-Lox recombinase technology circumvents these issues and allows conditional gene knock-out in a time and cell-type specific nature.

4.2.3 Cre-Lox technology

Cre (cyclisation recombinase) is an enzyme derived from the bacteriophage P1, that excises DNA sequences flanked by “LoxP” sites: unique 34 base-pair
nucleotide sequences that serve as points for recognition and reintegration, allowing Cre to ligate the DNA back together [180]. The Cre-Lox system was developed for use in eukaryote organisms throughout the 1990s [181] and is now a well-established method for achieving conditional, cell-type specific gene deletion.

For inducible cell-specific gene deletion, two transgenic constructs are required to be integrated using homologous recombination. One contains the gene of interest flanked by LoxP sites (floxed), whilst the other must have Cre recombinase fused with a promoter region that is hormone (eg. testosterone) or drug-sensitive (eg. doxycycline). When both alleles co-exist in the same cell, and in the presence of the specific inducing factor, active Cre recombinase is expressed and the gene of interest excised (Figure 19).

![Figure 19. Cre-Lox mouse breeding](image)

Mice with the Cre protein expressed in a specific cell type are bred with mice that contain a gene of interest flanked by LoxP sites. When the mice are bred, cells expressing Cre will have the target gene excised. Reproduced from [182].

Although commonly used for gene deletion, *de novo* gene expression can also be achieved with the Cre-Lox system. If a transgene is fused with a ubiquitous promoter and a floxed STOP sequence, active Cre removes the STOP insertion, permitting expression of the new gene (Figure 20). This technique is frequently used to create “reporter” cells or organisms that allow rapid identification of successful transgene integration and expression (eg. LacZ or yellow fluorescent protein (YFP)).
Figure 20. Cre-Lox construct for de novo gene expression

The STOP sequence is a short sequence of transcriptional STOP codons or terminators that prevent gene transcription. Activated Cre excises the STOP sequence allowing gene transcription and expression. Reproduced from [182].

Cre activity is very efficient and the main limitation to this system is promoter specificity, as very few are perfectly cell-type specific and even low levels of Cre expression can lead to recombination [183].

4.3 Study design

4.3.1 Kidney androgen promoter 2i improved Cre (KAP2iCre)

The kidney androgen-inducible protein (KAP) was first identified by in vitro translation of male mouse kidney mRNA in 1979 [184]. The function of this 20 kiloDalton (KD) protein remains unknown, but in-situ hybridisation studies show highly-specific proximal tubular expression and exquisite androgen sensitivity [185]. In comparison to normal males, female and castrated male mice express 10 and 200 fold less KAP mRNA respectively.

The KAP2iCre (KC) construct was developed and used by the original investigators through their work assessing the role of renin and angiotensin expressed within the proximal tubule [172, 186]. By fusing regulatory elements of both the KAP promoter and angiotensinogen genes, enhanced high level, PTC-specific, codon-optimised Cre (iCre) expression could be achieved with administration of exogenous testosterone (without functional KAP or angiotensinogen production). Low level Cre expression was detected in brain and liver extracts using RNase protection assays, although <1% of hepatocytes stained positively in their LacZ reporter mouse. The same group
has since published work using the KC construct to either overexpress or deplete the proximal tubular angiotensin receptor type-1A (AT1A), resulting in an increase and decrease of systemic BP respectively, independent of testosterone administration alone [187].

The KC transgenic mouse is commercially available and was purchased from the Jackson Laboratory, Maine, USA (strain name: B6.Cg-Tg(Kap-cre)29066/2Sig/J). Male KC+ mice exhibit variable levels of KC expression during adulthood due to endogenous testosterone production whereas female mice do not [172]. To achieve complete temporal control over KC expression, **only female mice were used for experiments** (see final discussion). Administration of a subcutaneous testosterone pellet for a minimum of ten days [186] was necessary to induce functional KC expression and subsequent floxed gene deletion - evidence of which was examined through expression analysis of genes, proteins and enzyme activity.

Two KC double transgenic mice were bred: the first, a ROSA26eYFP reporter mouse to confirm cell-type specific KC induction with exogenous testosterone, the second, a DDAH1-floxed mouse designed to test the study hypothesis (Figure 21).

![Figure 21. KAPiCre mediated YFP expression and DDAH1 deletion](image)

Following 10 days of exogenous testosterone treatment in female mice, expression of renal proximal tubular cell-specific active KAPiCre causes recombination events between loxP sites and excision of intervening gene sequences. **A**, the YFP reporter mouse and **B**, the PT-specific DDAH1 knock-out (PTD1KO) mouse.
4.4 The ROSA26eYFP reporter mouse

ROSA26 is a gene locus on murine chromosome 6 that is widely expressed in different cell-types but its function is unknown. It was first identified in mouse embryonic stem cells (ECs) in 1991 [188] and has since been used as a locus for the insertion of transgenic constructs through homologous recombination to generate over a hundred transgenic mouse lines. Advantages of targeting ROSA26 over other methods include its reliable success in transgene insertion, wide tissue expression under its own promoter and absence of detectable downstream effects when in the heterozygote state [189].

This particular strain of ROSA26 transgenic mouse contains an enhanced yellow fluorescent protein (eYFP) inserted into the locus. Constitutive eYFP expression is blocked by an upstream floxed STOP sequence that requires excision by active Cre before it can be transcribed. A mouse expressing both the ROSA26eYFP and KC transgenes would provide a tool for confirming the induction of PTC-specific iCre expression using exogenous testosterone.

A male ROSA26eYFP heterozygous mouse on a mixed B6/129 background was a kind gift from Professor Graham Williams, Imperial College, London. Animal genotyping was performed using two separate primer mixes; one for KC, a second for ROSA26eYFP transgene (primer sequences are listed in the Appendix).

4.4.1 Gross phenotype

4.4.1.1 Breeding

Mice heterozygous for ROSA26eYFP (mixed B6/129 background) and KC (C57BL/6J background) were successively bred to produce offspring that were normal in size, behaviour and fertility with litter genotype ratios consistent with Mendelian inheritance. First generation female mice heterozygous for both KC and ROSA26eYFP were used at 6-10 weeks of age.
4.4.2 Generalised effects of exogenous testosterone

Administration of a subcutaneous testosterone pellet for a minimum of ten days was necessary to induce functional KC expression and subsequent floxed gene deletion. Mice treated with testosterone gained more weight and displayed more aggressive behaviour to handling than their untreated counterparts.

4.4.3 Kidney-specific induction of KC transcription

Following testosterone treatment, significant KC mRNA transcription was detected in kidney tissue (p=0.02) from mice containing the KC allele but not in the absence of testosterone or the KC transgene (Figure 22). KC transcription was undetectable in organ tissue apart from the brain, although this was statistically insignificant (p=0.06).

![Figure 22. Testosterone induction of KC mRNA transcription](image)

Values are corrected for genomic DNA (gDNA) using a non-reverse transcribed control of the same sample. This was necessary to discriminate transcribed KC messenger RNA (mRNA) from KC sequences in contaminating genomic DNA. Corrected values obtained in KC- tissue represent non-specific or contaminant polymerisation (occurring at Ct values >35). Significant KC transcription was observed only in KC+ mouse kidney tissue (1.89 AU; 95%CI -2.1-5.8) when compared to other KC+ organs; Two-way ANOVA with Bonferroni’s post test, n=4, **p<0.01.

4.4.4 Kidney-specific induction of YFP protein expression

YFP has significant amino acid sequence homology with green fluorescent protein (GFP) to allow detection using a GFP antibody. Western blot analysis revealed YFP in kidney tissue from testosterone treated double transgenic
mice and not in other organs or kidney tissue from testosterone naïve mice (Figure 23).

![Western blot analysis of whole organ lysates for YFP expression](image)

**Figure 23. Western blot analysis of whole organ lysates for YFP expression**

YFP expression is limited to the kidney of KC-ROSA26eYFP transgenic mice treated with testosterone (arrows). No expression was detected in other organs or testosterone naïve mice. Pure GFP protein was used as a positive control (27kDa). GFP antibody performance was variable; non-specific bands a frequent limitation as in (C). Three different antibodies were used to confirm findings; A, Abcam (ab6556); B, Clontech; C, Abcam (ab290).

4.4.5 Proximal tubule-specific expression of YFP in kidney sections

4.4.5.1 Direct fluorescence microscopy

Fluorescence microscopy was used to identify inducible YFP expression within tubular structures, comparing kidney sections taken from testosterone treated and untreated double transgenic mice.

![Fluorescence microscopy of KC-ROSA26eYFP kidney tissue](image)

**Figure 24. Fluorescence microscopy of KC-ROSA26eYFP kidney tissue**

Autofluorescence of tubular structures obscures any reliable detection of YFP expression in proximal tubules. Sections from both testosterone untreated (A) and treated animals (B) appear the same. i, DAPI spectrum; ii, yellow spectrum; iii, merged image. Bars = 200 µm.
Immunohistochemistry was not performed in these studies – detection relied upon the endogenous fluorescence of YFP. Initial assessments were restricted by significant yellow-spectrum autofluorescence within the tubules, thus regardless of testosterone treatment and successful transgene activation, visual detection of YFP expression was not possible (Figure 24).

4.4.5.2 Fluorescent peak correction

To remove interfering tubular autofluorescence, the emission spectra from multiple user-defined regions across the kidney tissue were scanned using Leica LAS Lite Viewer software. Two overlapping emission peaks were identified within the filtered yellow spectrum; one at approximately 527 nm corresponding to eYFP, the other at 540nm, representing the non-specific background autofluorescence detected in initial fluorescence imaging. The selectivity of the fluorescent filter limits the precision of observed emission spectra, however Leica software allows a more focussed window of emission detection to be defined digitally, subtracting unwanted fluorescence (Figure 25).

Using this method, YFP fluorescence was confirmed in kidney tissue sections from testosterone-treated mice and not in those untreated.
Furthermore, the tubular YFP expression was heterogeneous, evident in portions morphologically consistent with the proximal tubule and not in other tubular segments (Figure 26).

![Image](image-url)

**Figure 26. Digital subtraction for YFP detection in kidney sections**

In each row, the same section images are displayed before and after digital subtraction of the background emission peak. In testosterone-treated mouse tissue, (A) YFP is difficult to distinguish from background autofluorescence, but after digital subtraction of background (B), YFP is distinguished. Kidney tissue from testosterone-naïve mice revealed no detectable YFP before (C), or after digital subtraction (D). Bar = 50 µm (x10 magnification).

4.4.6 Tubule-specific YFP expression in single tubular cells

To support the findings from microscopic examination of KC-ROSA26eYFP renal tissue, a single cell-imaging approach (ImageStream) was used to confirm successful induction of the transgene, specific to renal tubular cells. Renal tubular fractions were isolated from testosterone treated and untreated KC-ROSA26eYFP mice as previously described. Single cell imaging of
tubular cells extracted from testosterone treated mice, revealed a population of YFP-expressing cells that was not detectable in tubular fractions extracted from untreated mice (Figure 27).

Figure 27. ImageStream single cell analysis of extracted tubular cells from KC-ROSA26eYFP mice  
A, testosterone untreated. B, testosterone treated. The first gating analysis; R2, sorts cells according to spherical shape (discounting asymmetric, non-cellular material). The second gating analysis; R4, identifies cells fluorescent in the yellow spectrum. In A (untreated), no R4 gated-YFP-expressing cells are identified. In B (treated), a population of YFP-expressing cells are detected and imaged in C.
4.5 The proximal tubule-specific DDAH1 knock-out mouse (PTD1KO)

In order to control for the effect of testosterone treatment alone, three experimental groups were necessary:

1) No testosterone KAPiCre + (T-KC+)
2) Testosterone KAPiCre - (T+KC-)
3) Testosterone KAPiCre + (T+KC+; PTD1KO)

Some results are presented with two groups for clarity (2 and 3); the KC- and KC+ groups, where both have been exposed to testosterone.

4.5.1 Breeding

Male mice heterozygous for KC on a C57BL/6J background were bred with transgenic DDAH1-floxed mice on a mixed strain background (generated previously by our own group). In this construct, LoxP sites were inserted to flank exon 1 of DDAH1 (encoding the initiating methionine residue and the first 100 amino acids of the protein).

Successive breeding achieved the breeding genotypes required for production of experimental animals; all homozygous for the DDAH1-floxed transgene (D1) but only one parent being a KC heterozygote, the other not possessing the KC transgene. In this way, double transgenic, KC positive (KC+) “test” animals were bred alongside single transgenic, KC negative (KC-) littermate controls. Cohorts of mice for each study derived from the same grandparent lineage.

4.5.2 Gross phenotype

4.5.2.1 Untreated transgenic mice

Mice possessing one or both of the KC or D1 transgenes were normal in size, behaviour and fertility with litter genotype ratios consistent with Mendelian inheritance.

4.5.2.2 Generalised effects of exogenous testosterone

For the purposes of physiological and disease model testing, PTD1KO mice were exposed to testosterone for longer periods than the ROSA26eYFP
reporter mice, which accentuated the effects of testosterone on weight gain and aggression previously seen with the reporter mouse (Figure 28).

Figure 28. The effect of testosterone treatment on body weight
After 21 days, body weight increased significantly more in mice treated with testosterone compared to those untreated; T-KC+ vs T+KC- and T+KC+, 6.6 vs 19.1 and 22.2 % (95% CIs 4.6-8.5, 14.5-23.8 and 18.6-25.8; p<0.05 and p<0.01 respectively). There was no statistical difference between T+KC- and T+KC+ mice. One-way ANOVA with Bonferroni’s post test, n=6 for each group, *p<0.05 **p<0.01.

After a minimum of 2 weeks of testosterone treatment, ~10% (8 of 81) of mice developed utero-vaginal (UV) prolapse necessitating sacrifice, whilst a further proportion developed variable degrees of genital hypertrophy that did not require intervention (Figure 29). These testosterone treatment effects have been reported previously [190] and were unaffected by genotype.

Figure 29. Genital hypertrophy (A) and UV prolapse (B) in female mice treated with testosterone. (UV, uterovaginal).

4.5.3 Kidney-specific induction of KC transcription
Kidney-specific induction of KC mRNA transcription previously seen in the reporter mouse was confirmed in PTD1KO mice, with no expression in other
organisms. Within whole kidney, testosterone treatment induced KC transcription 4000-fold (p<0.01) in KC+ mice compared with background signal in KC- mice. The transcription of KC in mice naïve to testosterone was detected but 29-fold less than treated mice (p<0.001) and statistically insignificant when compared to KC- controls (Figure 30).

Figure 30. Whole kidney KC mRNA transcription in PTD1KO mice
Values are corrected for genomic DNA (gDNA) using a non-reverse transcribed control of the same sample. This was necessary to discriminate transcribed KC messenger RNA (mRNA) from KC sequences in contaminating genomic DNA. Corrected values obtained in KC- tissue represent non-specific or contaminant polymerisation (occurring at Ct values >35). KC+ mice showed induction of KC transcription in response to testosterone; T-KC+ vs T+KC+, 0.02 vs 0.63 AU (95% CI -0.03-0.07 and 0.27-1.0; p<0.01). Testosterone naïve mice (T-KC+) did not express significantly more KC mRNA than KC- counterparts; 0.02 vs 0.0002 (95% CI -0.0002-0.0004). One-way ANOVA with Bonferroni’s post test, n=6 in each group, **p<0.01.

4.5.4 Kidney-specific induction of DDAH1 deletion

Examination of kidney tissue from KC+ mice treated with testosterone revealed evidence of DDAH1 gene deletion and subsequent reductions of mRNA, protein and enzymatic activity. Tissue from KC- mice showed no evidence of DDAH1 deletion.

4.5.4.1 Gene

Following testosterone treatment, qualitative end-point PCR (epPCR) analysis of whole kidney tissue confirmed genetic DDAH1 deletion in KC+ mice with evidence of deletion to a lesser extent, in the liver and brain (Figure 31A).
Figure 31. Organ-specific DDAH1 gene deletion in testosterone-treated mice

(A) End-point PCR. Primer sets were designed to identify the presence of KC and floxed DDAH1 transgenes, but also recombined sequences, signifying DDAH1 deletion. Only KC+ mice exhibited evidence of floxed DDAH1 excision in the kidney and to a lesser extent, the liver and brain. (B) RT-qPCR. Reduced DDAH1 mRNA transcription was confined to the kidney; KC- vs KC+, 2.57 vs 1.22 AU (95% CIs 1.91-3.23 and 0.36-2.08; p<0.05). Comparisons between KC- (white bars) and KC+ (black bars) within each organ (DDAH1 expression is organ specific). Mann-Whitney U test, n=6 in each group, *p<0.05.

Quantitative (RT-qPCR) analysis revealed a ~50% reduction of DDAH1 mRNA transcription in KC+ mice, significant only in kidney tissue (p<0.05) and not other organs (Figure 31B).

4.5.4.2 Protein

Western blot analysis of whole kidney tissue revealed ~80% less DDAH1 protein in KC+ mice compared to KC- mice (p<0.001; Figure 32A).

4.5.4.3 Enzyme

DDAH1 enzyme activity measured in whole kidney homogenates was reduced by ~70% in KC+ mice compared to KC- mice (p<0.05; Figure 32B).
Figure 32. Kidney DDAH1 protein expression and enzyme activity

(A) Whole kidney DDAH1 protein expression was significantly reduced in KC+ mice; KC- vs KC+, 0.22 vs 0.04 AU (95% CIs 0.17-0.27 and 0.02-0.06; p<0.001). (B) Whole kidney lysate incubated with deuterium-labelled ADMA allowed LC-MS/MS measurement of enzymatically produced deuterium-labelled citrulline. DDAH1 activity was reduced in KC+ mice; KC- vs KC+, 991 vs 286 TIC (95% CIs 432-1550 and 7-565; *p<0.05). (GAPDH, glyceraldehyde 3-phosphate dehydrogenase; D7 citrulline, 7-deuterated citrulline; TIC, total ion count). Mann-Whitney U test, n=6 (KC-) and n=8 (KC+), *p<0.05 ***p<0.001.

4.5.5 Tubular-specific DDAH1 deletion

Quantification of KC and DDAH1 mRNA transcription in tubular fractions isolated from mice treated with testosterone reflected findings from examination of whole kidney: significant KC induction (p<0.01) with consequent ~75% reduction of DDAH1 mRNA transcription (p<0.05) (Figure 33).

Figure 33. KC and DDAH1 mRNA transcription in isolated renal tubules

Tubules were isolated from transgenic mice using magnetic bead separation as described in Chapter 10. (A) KC transcription was induced in KC+ mice; KC- vs KC+, 0.01 vs 3.56 AU (95% CIs 0.003-0.03 and 0.83-6.29; p<0.01). (B) DDAH1 mRNA transcription was reduced; KC- vs KC+, 14.5 vs 3.45 AU (95% CIs 5.1-23.9 and 1.6-5.3; *p<0.05). Mann-Whitney U test, n=6 for both groups, *p<0.05 **p<0.01.
4.6 Discussion

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4.6.1 Successful PT-specific gene-targeting

In response to testosterone treatment, activation of KAPiCre (KC) resulted in PT-specific YFP expression in the reporter mouse and DDAH1 deletion in the PTD1KO mouse.

To investigate the role of DDAH1 activity in the kidney during health and disease, this novel transgenic mouse has considerable advantages over previously created experimental animals that possess global DDAH1 deletion or overexpression. Global DDAH1 KO mice have elevated circulating ADMA and systemic BP rendering them impractical in a study aimed at differentiating between effects of systemic and local renal perturbations of the NO-ADMA-DDAH axis. Similarly, genetic overexpression is not tissue-specific and includes cells that do not normally express DDAH1 such as macrophages, which may well impact upon their responses to inflammation [102]. In addition, genetic or viral over-expression of DDAH1 activity to supra-physiological levels will not be subject to normal regulatory and adaptive responses, which may confound results further.
This novel transgenic mouse allows DDAH1 deletion that is not only PT cell-specific, but is conditional, thus avoiding adaptive changes that result from constitutive gene deletion from an early developmental age.

4.6.2 Limitations

Evidence of floxed DDAH1 recombination was detected in brain and liver tissue through qualitative PCR analysis, although quantitative RT-qPCR analysis demonstrated this to be statistically insignificant. These findings are consistent with previous reports from the original developers of the transgenic construct which describe KC expression in brain and liver tissue using RNA-protection assays, however only a negligible proportion of hepatocytes (<1%) stained positive in their LacZ reporter mouse [172]. As will be seen, in the absence of baseline alterations to BP or circulating ADMA, it is unlikely that negligible neuronal or hepatic DDAH1 deletion would impact upon renal function or disease progression.

Ideally, evidence of YFP expression or DDAH1 deletion would be demonstrated alongside cell markers that define the PT cell type. Immunological counter-staining in kidney sections or FACS analysis was attempted using Aquaporin-1 and megalin antibodies although these were unsuccessful despite numerous attempts. However, the PT constitutes the majority (~65%) of total renal mass [191] and the largest proportion of tubular cell mass. Fluorescence microscopy of kidney cortex identified YFP expression in a majority of tubules that morphologically represented PT segments. Further still, the technique for tubular isolation yields PT cells far in excess of 65% purity (see In vitro studies Chapter 10) and ImageStream single cell imaging identified YFP expression to suggest that the PT was successfully targeted.
5  **DOWNSTREAM EFFECTS OF PTD1 DELETION**

5.1  **Introduction**

Others have previously shown that global genetic DDAH1 deletion results in accumulation of ADMA and a subsequent fall in NO activity [105, 119]. In PTD1KO mice, it was anticipated that effects on the ADMA-NO axis would be confined to the kidney and perhaps, more specifically to the tubular compartment. Furthermore, if renal DDAH1 contributes significantly to renal tubule NO activity, its deletion would have significant downstream physiological effects upon tubular metabolic function and reabsorption (see Introduction Chapter 1.7: NO signalling in the kidney).

5.2  **Study design**

Mice used in any one study shared the same grandparent ancestry. In order to control for the effect of testosterone treatment and DDAH1 deletion, three experimental groups were necessary:

1)  No testosterone  KAPiCre +  (T-KC+)
2)  Testosterone    KAPiCre -  (T+KC-)
3)  Testosterone    KAPiCre +  (T+KC+; PTD1KO)

Some results are presented with two groups for clarity (2 and 3); the KC- and KC+ groups, where both have been exposed to testosterone.

Cohorts of PTD1KO mice and controls were treated with testosterone pellets for 10 - 17 days and plasma, urine and kidney tissue was analysed for MAs, NOx and biochemical composition. Urinary proteomics provided an additional approach to phenotype analysis whilst a separate study measured the haemodynamic effects of PTD1 deletion using invasive blood pressure monitoring under terminal anaesthesia.
5.3 Results

5.3.1 Methylarginine (MA) and NO content

5.3.1.1 Isolated tubules

Comparison of tubular fractions isolated from KC- and KC+ mice revealed almost 7-fold higher intracellular ADMA (p<0.05) with PTD1KO and a corresponding 2.5-fold reduction in stable end products of NO activity (nitrites and nitrates; NOx; p<0.05) (Figure 34).

**Figure 34. Effect of PTD1KO on tubular MA and NO**

Tubules were isolated from transgenic mice using magnetic bead separation as described in Chapter 10. (A) Isolated tubules from PTD1KO mice (KC+) had significantly raised ADMA; KC- vs KC+, 14.0 vs 93.52 pM/mg (95% CIs 3.6-24.39 and 10.4-176.7 respectively; p<0.05) whilst (B) NOx was lower; 4.55 vs 1.82 µM/mg (95% CIs 1.93-7.17 and 0.38-3.26 respectively, p<0.05). SDMA is not a substrate for DDAH1 and consistent with this, was unaffected by DDAH1 deletion. Mann-Whitney U test, n=6 in each group, *p<0.05.

5.3.1.2 Whole kidney, urine and plasma

MAs and NOx levels in whole kidney lysates, urine and plasma were not significantly altered following PTD1KO (Figure 35).
Figure 35. Effect of PTD1KO on systemic MA and NOx

Examination of whole kidney lysates (A, B), urine (C, D) and plasma (E, F) revealed no significant (ns) alteration in MA or NOx concentrations between KC- and KC+ animals. Mann-Whitney U test, n=6 in each group.

5.3.2 Kidney NO signalling enzymes

PTD1KO did not lead to significant compensatory alterations of other NO signalling enzymes; DDAH2, AGXT2 and NOS isoforms (Figure 36).

5.3.3 Urinary profiles

NO signalling influences renal tubular cell re-absorptive and secretory function (Chapter 1.7.2). Tubular dysfunction in humans can be detected in the clinical setting with urinary leak of essential metabolites including phosphate, calcium, glucose, amino acids, low molecular weight proteins and lactate [192, 193].
The effect of PTD1KO upon these metabolites was assessed. Given the initial findings of a direct effect of exogenous testosterone upon urine volume and proteinuria, the untreated group (T-KC+) is also represented.

5.3.3.1 Volume and proteinuria

Urinary volumes were elevated in mice treated with testosterone but only to statistical significance in PTD1KOs (p<0.05; Figure 37A). Testosterone treatment reduced urinary creatinine 2-fold (p<0.01) and increased proteinuria 4 to 5-fold with no additional effect from DDAH1 deletion (Figure 37 B, C). These changes were reflected in calculated urine protein:creatinine ratios (Figure 37D).
Figure 37. Effect of testosterone and PTD1KO upon urine volume and proteinuria

Urine was collected over 24 hours in metabolic cages. Comparisons made between T-KC+ and other groups. (A) Urine volume T-KC+ vs T+KC- and T+KC+; 37 vs 57 and 64 µL/g/24hrs (95% CIs 24-50 vs 42-75 and 49-78; p>0.05 and p<0.05 respectively). Urinary creatinine (B) and protein (C) was measured by NHS laboratory biochemical automated analysis and verified by LC-MS/MS and Bradford’s assay respectively. UPCR was calculated from these results (D). Testosterone treatment increased UPCR with no additional effect of DDAH1 deletion; T-KC+ vs T+KC- and T+KC+; 0.36 vs 3.1 and 2.83 g/mM (95% CIs 0.21-0.5 vs 1.6-4.6 and 2.3-3.4; both p<0.001). One-way ANOVA with Bonferroni’s post test. T-KC+ n=7; T+KC- n=8; T+KC+ n=20. *p<0.05 **p<0.01 ***p<0.001.

5.3.3.2 Urinary electrolytes

With the exception of sodium in testosterone treated mice, there were no significant changes detected in urinary electrolyte concentrations. DDAH1 deletion tempered this effect rendering it non-significant (Figure 38).
Figure 38. Urinary electrolytes following testosterone treatment and PTD1KO

(A) Only urinary sodium was statistically different (elevated) in testosterone treated mice. DDAH1 deletion made this non-significant. T-KC+ vs T+KC- and T+KC+; 34 vs 56 and 49 mM/mM (95% CIs 26-43 vs 42-70 and 40-52; p<0.05 and >0.05). One-way ANOVA with Bonferroni’s post test. T-KC+ n=7; T+KC- n=8; T+KC+ n=20. *p<0.05.

5.3.3.3 Urinary glucose and lactate

Both urinary glucose and lactate were elevated in PTD1KOs (~50% and 3-fold respectively compared with KC- mice) although due to high variability, these increments did not reach statistical significance (Figure 39).
Figure 39. Urinary glucose and lactate following testosterone treatment and PTC DDAH1 deletion

(A) Increases in urinary glucose and (B), urinary lactate were statistically insignificant. One-way ANOVA with Bonferroni’s post test. T-KC+ n=7; T+KC- n=8; T+KC+ n=20.

5.3.3.4 Urinary amino acids

Urinary L-arginine was reduced by testosterone treatment alone (3-fold) but not in PTD1KOs. Testosterone treatment produced a non-significant rise in urinary concentrations of L-citrulline and L-glutamine with no effect of DDAH1 deletion (Figure 40).

Figure 40. Urinary amino acids

(A) Urinary arginine decreased by testosterone treatment but increased by DDAH1 deletion. T-KC+ vs T+KC-; 0.142 vs 0.045 AU (95% CI 0.1-0.183 and 0.028-0.062); p<0.05. T+KC- vs T+KC+; 0.045 vs 0.126 (95% CI 0.049-0.204); p<0.05. Urinary citrulline (B) and glutamine (C) were not significantly altered. One-way ANOVA with Bonferroni’s post test. T-KC+ n=7; T+KC- n=8; T+KC+ n=20. *p<0.05.
5.3.4 Urinary proteomics

Urinary proteomic analysis was performed as part of a collaborative work with Drs Pedro Cutillas and Peter Faull in the Proteomics department of the MRC Clinical Sciences Centre, London. Mass spectrometry was used to determine the effect of PTD1KO upon peptide profiles in urine. Any effect would represent altered proximal tubular peptide handling in terms of; failure to reabsorb; increased secretion or expulsion into the urinary space. Data analysis was performed by Dr Cutillas and Sanjay Khadayate (bioinformatician, MRC Clinical Sciences Centre, London).

A total of 1437 peptide fragments were detected and identified according to delta times and mass to charge ratio (m/z). Comparison of urinary peptide profiles in KC- and KC+ mice (PTD1KO), revealed a total of 84 significant differences according to a p value of <0.05. Figure 41 represents the top twenty most altered peptide fragment abundances and the genes from which they originate, both decreased and increased with PTD1KO expression respectively.
Figure 41. Heat map of urinary proteomic analysis in PTD1 deletion at baseline

82 of 1057 peptides screened were significantly altered by DDAH1 deletion. The ten most significantly down- and upregulated peptides are represented. (q values represent values of significance adjusted for multiple comparisons). n=4 as represented.
5.3.5 Haemodynamics

Invasive terminal BP recording in adult female mice (aged 8-10 weeks) revealed reproducible trends for elevated BP with testosterone treatment and reduced BP in response to PTC-DDAH1 deletion although neither effect was statistically significant (Figure 42).

Figure 42. Systemic BP and heart rate in response to testosterone treatment and PTC-specific DDAH1 gene deletion
Measured by carotid artery cannulation under terminal anaesthesia. A trend for a rise in BP with testosterone treatment and a reduced BP in response to PTD1KO was observed but neither effect was statistically significant. (A) diastolic BP; (B) systolic BP; (C) mean arterial pressure (MAP); (D) heart rate. One-way ANOVA with Bonferroni’s post test, T-KC+ and T+KC- n=6, T+KC+ n=8.
5.4 Discussion

**Key findings**

**PTD1KO downstream effects include**

- Tubule-specific ADMA accumulation (7-fold) and NO reduction (2.5-fold)
- No alteration of ADMA/NO content in plasma, urine or whole kidney
- No alteration of urinary electrolytes, glucose or lactate
- Increased urinary arginine but not citrulline or glutamine

- Significantly altered concentrations of urinary peptides (84 of 1437 measured)
  - Uromodulin reduction (8.5-fold)
  - Collagen type 1 subunit reduction (6.5-fold)

**Direct effects of testosterone treatment include**

- Raised urinary volumes (50%)
- Reduced urinary creatinine (50%)
- Raised urinary protein (5-fold)

5.4.1 A novel tool for investigating NO signalling in the PT

As anticipated, PTD1KO deletion had direct effects upon the ADMA-NO axis in tubular cells that were absent systemically. Significantly elevated tubular cell ADMA and consequently, reduced NOx concentrations were not reflected in whole kidney, urine or plasma, which represents the cell-type specificity of PTD1 deletion. Global DDAH1 deletion has been achieved previously, but this is the first transgenic mouse with renal PT-specific DDAH1 enzyme deletion, thus providing a valuable tool with which to investigate the role of NO signalling in PT function.

To date, investigation of the role of NO signalling in PT function has been limited to the use of pharmacological manipulation in microperfusion and cell culture experiments. Considerable limitations exist with these approaches. The highly variable and often divergent effects of NO manipulation likely manifest from doses of NOS inhibitors or NO donors that are non-physiological to the PT *in vivo*. Furthermore, both techniques create an artificial environment (altering for example; temperature; oxygen tension; pH; neurohumoral triggers) in which normal adaptive responses may well be compromised if not completely abrogated.
5.4.2 A lack of downstream effects?

Perversely, these same adaptive mechanisms may in part, have contributed to the lack of phenotype witnessed in this series of studies. PT function assessed by urinary metabolites remained largely intact, albeit with some increase suggested in urinary loss of glucose and lactate (although not statistically significant). Increased urinary arginine (but not citrulline or glutamine) in PTD1KOs only rescued a lowering effect of testosterone treatment, suggesting a build-up of NO substrate rather than inhibited PT reabsorption of amino acids. Although a reproducible trend for lowered systemic BP in PTD1KOs was observed, this remained statistically insignificant across repeated studies.

The mice in this study were treated with testosterone for a minimum of ten days as indicated by the original developers of the KAPiCre construct [186], in order to ensure successful KAPiCre activation. All mice were sacrificed for biological phenotyping within a week (between 10-17 days’ exogenous testosterone treatment). This time period of treatment was chosen in an effort to make an assessment of the direct effects of reduced PT cell DDAH1 activity. Longer periods would conceivably introduce confounding effects of downstream adaptations and prolonged testosterone exposure (discussed further in Chapter 9).

5.4.3 Urinary proteomics: uromodulin

Interestingly, urinary uromodulin (UMOD) was reduced >8-fold in the PTD1KO mouse. As UMOD is known to be exclusively expressed in epithelial cells of the thick ascending limb [194], this effect indicates that functional changes are communicated from PT epithelial cells, downstream along the nephron to those in the Loop of Henle. What is not clear is whether increased ADMA in the tubules of PTD1KO mice is the chief effector molecule, or an alternative molecule communicates the effect. The identification of 82 significantly altered peptides indicates that PT-specific NO signaling has far-reaching effects upon epithelial cells in different segments along the course of the nephron.

UMOD (or Tamm-Horsfall protein) is of particular interest as it has been associated with renal immunomodulation and the progression of CKD
UMOD is the most abundant mammalian urinary protein and despite being identified over 25 years ago [196], its function remains uncertain. Controversy exists as to whether UMOD is a predominantly, anti- or pro-inflammatory protein. *In vitro* and *in vivo* studies reveal protective effects against nephrolithiasis [197] and urinary tract infections by inhibiting crystal aggregation, and trapping bacteria [198], cytokines and cellular debris, to facilitate their excretion [199]. However, other *in vitro* experiments demonstrate that UMOD can stimulate proinflammatory cytokine release from whole blood [200] and facilitate neutrophil migration across epithelial monolayers [201].

The relevance of UMOD and other urinary peptides affected by PTD1KO is discussed further, in the final discussion (Chapter 12.6).

5.4.4 Limitations

Measurement of urinary total protein does not allow differentiation of glomerular (albumin leak) from tubular dysfunction (failure of reabsorption of amino acids and low molecular weight proteins). Attempts were made to assess failure of tubular reabsorption through measurement of urinary amino acids, glucose and lactate [193]. Alternative approaches to assess these relative effects could include measurement of both urinary total protein and albumin, along with retinol-binding protein [202]. Furthermore, had time allowed, an assessment of GFR using inulin clearance would have provided further insights into alterations of renal function secondary to PT cell DDAH1 deletion.

Many of the effects assessed in this study were reliant upon urinalysis. Significant limitations exist with the use of metabolic cages to collect urine. Urine drips down the walls of the cage into glass bottles that are cleaned only by water-rinsing between experiments. In addition, chow, drinking water overflow and faeces are unrestricted from falling into the collection vessels. Urine specimen contamination is therefore inevitable, producing highly variable results that identify only very large genotype or treatment effects.
5.4.5 Direct effects of testosterone

Due to the limitations in urine collection and analysis described above, it is difficult to interpret the effects of testosterone upon urinary volume, creatinine and protein. A significant increase in body weight gain was observed in testosterone-treated mice suggesting oral intake of food and water was increased. Such increased feeding activity could feasibly exacerbate the likelihood of urine collection vessel contamination discussed above. Testosterone-related polydipsia (thirst) or increased activity would increase drinking water drip contamination of urine collections and could alter urinary results, although this is not supported by urinary electrolyte concentrations that remained the same, irrespective of testosterone exposure - one would expect these to be diluted.

Tubular function (and particularly Na/K ATPase function, upon which the PT is highly dependent) is known to be under hormonal control [203]. Although the role of kidney androgen-sensitive protein (KAP) is yet to be determined, its very existence indicates that PT function is under androgenic influence. A recent report identified that transgenic overexpression of KAP developed hypertension and increased renal oxidative stress [204], although the KAPiCre transgenic construct used in this study does not produce functional KAP protein, so is unlikely to produce the same effect.

Protein in the urine originates from either glomerular leakage or altered tubular reabsorption/secretion of low-molecular weight proteins. Without quantifying albuminuria (to identify the contribution of glomerular protein leakage), it is impossible to say whether the proteinuric effect of testosterone (if genuine) is due to increased protein leakage into the ultrafiltrate, or dysregulated tubular function. A direct association of testosterone exposure and albuminuria has been confirmed in mouse studies using both gender and strain comparisons, which was further demonstrated by androgen supplementation in female mice [205].

Testosterone has direct effects upon NO production. Androgens have been demonstrated to have divergent effects on NO-independent vasodilatation across different vascular beds [206]. Equally confusing is the increased CV and renal disease in rodent models, however in human clinical
trials, higher androgen levels associate with better CV health [207]. The direct effects of oestrogens and fundamental differences between animal models of disease and human cohorts confound an understanding of the complex relationship between testosterone and NO-related disease.
6  **SALT-FEEDING IN PTD1KO MICE**

6.1  Introduction

PTD1KO characterisation studies demonstrated that disruption of proximal tubular DDAH1 elevated ADMA and in turn, reduced NO synthesis. Although NO signalling is known to regulate tubular sodium reabsorption and TGF [136-139] no effects upon BP or urinary electrolytes and volume were observed at baseline in the PTD1KO mouse. Encouragingly, functional downstream effects of DDAH1 deletion were confirmed through proteomic urinalysis showing significantly altered peptide profiles. A salt-feeding study was undertaken in an attempt to expose other downstream effects of tubular DDAH1 abrogation in a physiologically stressed environment using a high salt (sodium) load.

6.2  Study design

Published rodent salt-loading protocols vary in dose (2-8% sodium in chow) and duration from one week and beyond [208]. For practical ease and clarity, only two groups; KC- and KC+ mice were treated with testosterone (as previously described) and fed low- or high-sodium chow (0.03% or 3.15%) with ad libitum access to normal water. Standard chow has a sodium content of 0.1% (Harlan Laboratories Teklad Global Diet). The group numbers were as follows:

<table>
<thead>
<tr>
<th></th>
<th>KC-</th>
<th>KC+ (PTD1KO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low salt</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>High salt</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

Urine was collected in metabolic cages prior to and following 8 days of salt feeding. Invasive blood pressures were recorded under terminal anaesthesia and plasma and kidney tissue were harvested as described previously. Samples were analysed for evidence of altered NO signalling and renal sodium handling.
6.3 Results

6.3.1 MAME and NOS enzymes

Consistent with findings reported in Chapter 4, PTD1KO mice exhibited significantly reduced whole kidney DDAH1 mRNA transcription, protein and enzyme activity (Figure 43A-C). High salt feeding increased DDAH1 mRNA transcription (>50%; p<0.05) in KC- but not KC+ (PTD1KO) mice (Figure 43A). DDAH1 upregulation was not seen at the level of protein or enzyme activity however. Salt loading produced small increases in DDAH2, AGXT2 and iNOS transcription, although (with the exception of iNOS in KC- mice (~60%; p<0.05)), they did not reach statistical significance and no differences were observed between KC- and PTD1KO mice.

6.3.2 The NO-ADMA axis

Whole kidney ADMA content was higher in PTD1KO mice (~30%; p<0.01) but no additional effect of salt feeding was observed (Figure 44A). Of note, previous characterisation studies did not detect higher whole kidney ADMA content, it was restricted only to the isolated tubular fractions in which ADMA was elevated by ~60% compared with KC- controls. Consistent with elevations in DDAH1 and iNOS in KC- animals, both whole kidney and urinary NOx increased in response to salt feeding.

6.3.3 The effect of salt-feeding renal sodium handling

A high salt diet produced small, statistically insignificant (<5mmHg) increases in systemic BP with no differences between KC- and PTD1KO mouse strains (Figure 43). Appropriately in response to salt-loading, fractional excretion of sodium (FENa; the clearance of sodium adjusted for GFR) rose significantly (~25-fold; p<0.0001), whilst plasma aldosterone fell, which confirmed that significantly higher amounts of sodium were being ingested/excreted and serum aldosterone levels were appropriately suppressed (>2-fold; p<0.01). No additional effect of PTD1KO was observed. In animals fed a high salt diet, plasma sodium fell and plasma creatinine rose – only to a significant degree in animals with intact DDAH1.
Figure 43. Whole kidney tissue MAME and NOS isoform transcription

DDAH1 deletion is confirmed in these studies as previously shown. A, In DDAH1 intact (KC-) mice, salt feeding increased DDAH1 mRNA transcription by >50% (low vs high salt diet; 3.66 vs 6.47 AU; 95% CIs, 1.11-6.21 and 4.98-7.95 respectively; p<0.05). A high salt feed increased transcription of DDAH2, AGXT2 and iNOS but it did not reach statistical significance except iNOS in KC- mice (G; low vs high salt diet; 1.11 vs 1.84 AU; 95% CIs, 0.12-2.1 and 1.4-2.29 respectively; p<0.05). Two-way ANOVA with Bonferroni’s post test. Low salt KC- n=3, KC+ n=5; high salt KC- n=6, KC+ n=8. *p<0.05 **p<0.01 ***p<0.001.
Figure 44. Whole kidney and urinary ADMA and NOx content

A. Whole kidney ADMA is elevated in PTD1KO animals but with no additional effect of salt loading. Following a salt loading, KC- mice have significantly elevated kidney tissue (B, low vs high salt; 0.18 vs 0.24 mM/mg; 95% CI, 0.17-0.19 and 0.19-0.3; \( p<0.05 \)) and urinary NOx (D, low vs high salt; 0.37 vs 1.12 mM/mM; 95% CI, -0.39-1.11 and 0.17-1.49 respectively; \( p<0.05 \)) whereas PTD1KO mice do not. Two-way ANOVA with Bonferroni’s post test. Low salt KC- n=3, KC+ n=5; high salt KC- n=6, KC+ n=8. *\( p<0.05 \) **\( p<0.01 \) **\( p<0.001 \).

6.3.4 Tubular sodium channel expression

Western blotting analysis of whole kidney lysates taken from salt-fed mice was problematic due to poorly functioning antibodies, despite numerous attempts to optimise their efficacy. A high-salt diet appeared to inhibit NHE3 channel expression in all mice but no other significant effects were detected (Figure 46). RT-qPCR analysis was also performed to investigate sodium channel and Aquaporin 1 effects at the level of transcription. Although good replication was achieved with newly designed primers, no further insights were gained into diet or genotype effects (data not shown).
Figure 45. Systemic BP and renal sodium handling

A and E, Systemic BP (systolic and diastolic) was marginally elevated in mice fed a high salt diet (<5mmHg; ns). B, Urinary volumes were higher in high salt groups although variability rendered this non-significant. F, Fractional sodium excretion was raised in both mouse strains on a high salt diet (KC- low vs high salt: 0.28 vs 6.37%; 95% CIs -0.11 to 0.67 and 4.47-8.28 respectively; p<0.0001. KC+ low vs high salt: 0.24 vs 6.99%; 95% CIs 0.18-0.3 and 5.42-8.56; p<0.0001). G, Plasma aldosterone was lower in all high salt fed animals (KC- low vs high salt; 1097 vs 478 pg/mL; 95% CIs 842-3036 and 318-638; p<0.01. KC+ low vs high salt; 1350 vs 513 pg/mL; 95% CIs 1105-1596 and 375-652; p<0.0001). Only in KC- mice did plasma sodium fall (D) and creatinine (H) rise significantly (plasma sodium low vs high salt: 158 vs 152 mmol/L; 95% CIs 151-166 and 148-156; p<0.05. Plasma creatinine low vs high salt; 23 vs 27.6 µM; 95% CIs 15.7-30.1 and 24.7-30.4; p<0.05). Two-way ANOVA with Bonferroni’s post test. Low salt KC- n=3, KC+ n=5; high salt KC- n=6, KC+ n=8. *p<0.05 **p<0.01 ***p<0.001.

Fractional sodium excretion equation:

\[
FENa = 100 \times \frac{(\text{Urinary sodium} \times \text{Plasma creatinine})}{(\text{Plasma sodium} \times \text{Urinary creatinine})}
\]
Figure 46. Expression of tubular sodium channel protein in whole kidney

A. Cartoon illustrating expression patterns of renal tubular sodium channels and Aquaporin 1. B and C, representative Western Blots (WB) of sodium channel protein in whole kidney lysates. D, graphical representation of WB data. NHE3, sodium–hydrogen ion exchanger type 3; Na / Pi, sodium–phosphate cotransporter; NKCC, sodium-potassium-2chloride cotransporter; NCC, Na-Cl cotransporter; ENaC, epithelial sodium channel. Two-way ANOVA with Bonferroni’s post test. Low salt KC- n=3, KC+ n=5; high salt KC- n=6, KC+ n=8. *p<0.05.
6.4 Discussion

**Key findings**

**Effects a high salt diet**
- Increases kidney tissue DDAH1 transcription >50% in KC- mice only
- Does not affect whole kidney or urinary ADMA content
- Increases whole kidney and urinary NOx in KC- mice only
- Reduces plasma sodium in KC- mice only
- Increases plasma creatinine in KC- mice only

**Effects of PTD1KO**
- Limits salt-induced kidney tissue DDAH1 transcription
- Reduces salt-induced whole kidney and urinary NOx production
- Limits changes in plasma sodium and creatinine

Whole kidney tissue sodium channel protein expression was not affected by salt-feeding or PTD1KO

Salt-loading increased renal DDAH1 mRNA transcription in KC- mice and to a lesser extent, DDAH2, AGXT2 and iNOS. Consistent with this, renal and urinary NOx was elevated showing that NO activity was upregulated in response to salt-feeding. As previously discussed, NO signalling is important for renal sodium reabsorption and tubulo-glomerular feedback (TGF) through which tubular flow regulates glomerular filtration rate. Tubular upregulation of NO with salt-feeding indicates an adaptive natriuretic response to excrete excess sodium by inhibiting tubular reabsorption. Salt-feeding in rodents has previously been shown to significantly affect kidney NOS enzyme expression [209, 210]. Similar to microperfusion studies of NO regulating tubular reabsorption, the results are often contradictory. Acutely (within 48 hours), a high salt feed can upregulate renal nNOS and iNOS expression although at 3 weeks, this effect is reversed. The direction of shift in NO activity with salt-loading appears to be related to time of exposure, salt dose, level of associated hypertension and background genotype.

PTD1KO mice did not respond to salt-feeding by NO signalling enzyme upregulation like their KC- counterparts. Despite this, there was an absence of downstream phenotypic differences in terms of systemic BP, urinary volumes
or FENa. Possible explanations for a reduction in NO activity but little in the way of downstream effects include; no genuine difference in MAME or NOS gene expression or even NOx (a type I error); or a Type II error resulted and true genotype effects were not detected. This uncertainty originates largely from variable data from urine analysis (and its limitations – discussed previously and below) making detection of small adaptive downstream effects unlikely.

6.4.1 Limitations

6.4.1.1 Duration of exposure

Overall, results from the salt-feeding study were disappointing. Effects of the high salt diet alone did not reach the degree anticipated which inevitably contributed to the lack of significant effects observed with PTD1 deletion. Published rodent salt-loading protocols vary in dose (2-8% sodium in chow) and duration from one week and beyond [208], however it is possible that in these mice, 8 days of 3.15% sodium chow was inadequate to produce significant BP or biochemical changes.

In addition, gender-related effects may have contributed as only female mice were used in this study. In human studies at least, females exhibit increased salt-sensitivity compared to males when observing BP responses to altered dietary salt intake [211]. The administration of exogenous testosterone is likely to confound sex-related effects further, potentially reducing the degree of effect in this study.

6.4.1.2 Urine collection

Another important limitation is that of imprecise urine collection already discussed in the previous chapter. Urine collected in non-sterile metabolic cages is incomplete (evaporation, spilling) and inevitably becomes contaminated by the animal’s faeces and feed. This renders attempts at precise measurement of urinary electrolytes and volume futile, with a high potential for variable results and artefact for example; high sodium concentration from high-salt chow contamination or high urinary volumes from mice with polydipsia or leaking water bottles.

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6.4.1.3 Measuring sodium channel function

Although Western blotting has previously been used with success to determine adaptive sodium channel changes in the kidneys of NHE3 and NCC deficient mice [212], it is dependent upon effective antibodies and large alterations to allow successful detection. Furthermore, the degree of protein expression within a tissue lysate does not necessarily represent activity. Many post-translational modifications, including phosphorylation, protein kinase activation and membrane transmethylation, determine channel protein translocation to the apical membrane and sodium transport activity [213]. For identifying functional sodium transport effects of PTD1KO, microperfusion techniques could provide more reliable functional data that could then be supported by well-designed in vitro studies directly measuring channel flux (such as patch clamp techniques) [213].
7 MICROPERFUSION EXPERIMENTS

7.1 Introduction

Single nephron micropuncture was pioneered in the 1920s by Wearn and Richards (University of Pennsylvania) using glass micropipettes to cannulate single superficial glomeruli in frogs [214]. They demonstrated for the first time that protein-free fluid was filtered from the blood at the glomerulus and subsequently, by showing the presence of glucose and sodium chloride in blood and glomerular filtrate but not in bladder urine, they confirmed tubular reabsorption of these constituents.

From being restricted to sampling only superficial proximal and distal tubule portions, micropuncture techniques developed to access segments from the loop of Henle and collecting ducts through the renal papillae. A further advancement was microperfusion of a single tubule using oil droplets to isolate a segment and perfusing a prepared fluid with sample collection downstream [178]. This allows compounds of interest to be administered and their direct effects upon tubular reabsorption to be determined (Figure 47).

![Figure 47. Schematic aspects of micropuncture techniques](image)

1 demonstrates the collection of tubule fluid by a micropipette. 2 (perfusion pipettes) and 3 (collection pipettes) illustrate the means by which tubules may be continuously perfused. 4 illustrates the method of perfusing peritubule capillaries. These approaches may be used concurrently to determine tubular function in a defined artificial setting. (Reproduced from [178]).
Tubular fluid reabsorption ($J_v$) is calculated by the difference between volume ($V$) of artificial tubular fluid (ATF) perfusion ($\text{perf}$) and collection ($\text{coll}$) rates (nL/min) corrected by the length of tubule perfused (mm). The normal range for $J_v$ in a rat is $2.5 - 3.0$ nL/min/mm.

$$J_v = \frac{(V(\text{perf}) - V(\text{Coll}))}{PT \text{ length}}$$

To ensure an adequate proportion of ATF is recollected, non-reabsorbed C$^{14}$ inulin is perfused and >85% retrieval distally is deemed adequate.

Professors Chris Wilcox and William Welch at Georgetown University (Washington, USA) run a laboratory that performs renal tubule micropuncture techniques for investigating mechanisms of tubuloglomerular feedback and control of systemic blood pressure. Previously, in Sprague-Dawley rats, they demonstrated inhibited tubular reabsorption using ADMA administration and DDAH1 gene silencing (unpublished). To develop these interesting findings further, a collaborative investigation was set up to test the role of renal DDAH1 in proximal tubular sodium and fluid reabsorption.

Research assistant Miss Tracy Bell performed all of the microperfusion studies described here. The author visited the Georgetown laboratory to observe the technique and assist in the design of experiments using the specific DDAH1 inhibitor, L257 and transgenic PTD1KO mice; both developed and supplied by our group.

### 7.2 Effect of ADMA upon tubular reabsorption

This particular experiment was performed by members of Professor Wilcox's group, with no input from the author of this thesis. They are presented here for means of introducing the subsequent studies using L257 and the PTD1KO mouse, to which the author did contribute as described above.

Microperfusion with ADMA revealed dose-dependant inhibition of tubular fluid reabsorption (>30% at $10^{-4}$ M; Figure 48A). Synthetic NOS inhibitor, L-NG-nitro arginine methyl ester (L-NAME) was effective at lower
concentrations (10⁻⁶ M) but produced a similar maximal inhibition of reduction in Jv at higher concentrations (10⁻⁴ – 5).

In a separate series of experiments, systemic administration of DDAH1 silencing RNA (siRNA) reduced renal DDAH1 protein expression by ~50% and inhibited Jv by ~30% (Figure 48B).

**Figure 48.** Effect of ADMA microperfusion and DDAH1 gene silencing upon tubular reabsorption

(A) ADMA (10⁻⁴ M) or L-NAME (10⁻⁴ M) into the PT both reduced Jv significantly to 2.0±0.2 and 1.8±0.2 nl/min/mm, respectively (p<0.05). (B) siRNA was complexed with a polymer from TransIT in vivo gene delivery system. Rats received 100 µg of siRNA (or equivalent vehicle) in 6 mL of PBS via the left jugular vein as a 7-second bolus injection to mimic the protocol of “hydrodynamic stress” and 72 hours after IV injections micropuncture experiments were performed. Two-way ANOVA with Bonferroni’s post test. n=5. *p<0.05.

7.3 The effect of pharmacological DDAH1 inhibition upon tubular reabsorption

Novel selective inhibitors of DDAH were developed at UCL (London) [215]. NG-(2-methoxyethyl)-l-arginine (L257) and NG-(2-methoxyethyl)-l-arginine methylester (L291) are arginine derivatives unlike other potential DDAH inhibitors, as they do not exhibit interaction with the NOS or arginase enzymes. Both L257 and L291 inhibit DDAH activity in vitro with an IC₅₀ value of 20 µM (Figure 49), causing significant elevations in circulating ADMA levels when administered intravenously into rodents, and increase vascular tension when used in organ bath studies using mesenteric resistance vessels [105]. Successful DDAH inhibition is detected both in vivo and in vitro by raised ADMA levels, reduced NO, and their respective downstream functional effects. Additionally, in vitro DDAH activity assays can be performed in which
reduced L-citrulline production (from reduced ADMA metabolism) is observed. Because L257 and L291 inhibit DDAH through active site binding, the presence or expression of DDAH protein is unchanged.

![Graph showing percentage inhibition vs. compound concentration](image)

**Figure 49. Pharmacological DDAH1 inhibition using L257 and L291 in vitro**

DDAH activity was measured in rat kidney lysates in the presence of L-291 (black squares) and L-257 (black triangles) at the concentrations indicated. n=3. Reproduced from [105].

A pilot experiment was performed by perfusing L257 into superficial PT segments (anatomically most likely to represent S1 and S2 segments [216]) for 5-10mins at 20 nL/min, but this had no effect upon Jv (Figure 50). A working concentration of 100µM was used - derived from previously published studies (discussed and referenced above) wherein circulating ADMA was increased whilst systemic BP was unaffected in rodents. The maximum time period that the PT can reliably be perfused is approximately 20 minutes. This may be inadequate time for; tubular cells to absorb the compound; L257 to transit and bind to DDAH1; intracellular ADMA to accumulate causing competitive inhibition at the active site of NOS and block NO synthesis to eventually influence tubular fluid reabsorption.
Figure 50. Tubular reabsorption (Jv) in response to L257 administration

Short-term perfusion of L257 in the proximal tubule did not affect Jv; vehicle vs L257; 3.37 (± 0.2) vs 3.35 (± 0.6) nl/min/mm. The addition of an intravenous bolus of L257 2 hours prior to micropuncture significantly reduced Jv to 2.3 (±0.2) nl/min/mm compared with vehicle (p<0.01) and PT-L257 alone (p<0.05). One-way ANOVA with Bonferroni's post test. n=5.

Subsequently, an intravenous bolus injection of L257 (60 mg/kg) was administered 2 hours prior to micropuncture, which reduced Jv by ~30% (Figure 50). In addition, plasma and urine ADMA levels were elevated (45% and 10-fold respectively) with no significant change in L-NMMA or SDMA levels (Figures 51A and B). Concentrations were highly variable in ATF and although no significant differences were seen, a similar trend of increased ADMA was demonstrated (Figure 51C).

Figure 51. Plasma, urinary and tubular fluid MA content

Comparisons between PBS vehicle (white bars) and L257 (black bars) treated groups. (A) Plasma ADMA was increased following L257 treatment. Vehicle vs L257, 0.46 vs 0.67 µM (95% CIs 0.38-0.55 and 0.6-0.75; p<0.001). (B) Urine ADMA was also elevated following L257; vehicle vs L257, 1.06 vs 10.2 (95% CIs 1.01-1.1 and 4.4-16; p<0.01). (C) No significant changes were found in the MA content of ATF. Mann Whitney U test. n=5. **p<0.01 ***p<0.001.
In the preceding PTD1KO mouse characterisation studies, urinary amino acid and peptide profiles were influenced by PTD1 deletion. Urinary amino acid contents were measured in this study to determine the effect of L257 treatment on tubular handling of amino acids. Significant elevations of amino acids occurred in L257-treated rats; glutamine (4-fold; p<0.01), arginine (2-fold; p<0.05) although citrulline did not change. (Figure 52).

Figure 52. Plasma, urinary and tubular fluid amino acid profiles
(A) Plasma citrulline was significantly elevated (vehicle vs L257, 20.4 vs 29.2 µM; 95% CIs 16.8-23.9 and 26-32.3; p<0.001) and glutamine was not while arginine LCMSMS identification in these samples did not work despite repeated efforts. (B) Urinary glutamine and arginine acids significantly increased with L257 treatment (vehicle vs L257); glutamine, 0.64 vs 2.68 µM/mM creatinine (95% CIs 0.36-0.91 and 1.83-3.52; p<0.01); arginine, 0.17 vs 0.36 µM (95% CIs 0.04-0.29 and 0.21-0.5; p<0.05). (C) No significant changes were found in the amino acid content of ATF. Mann Whitney U test. n=5. *p<0.05 **p<0.01 ***p<0.001.

By blocking DDAH1 activity with L257, ADMA accumulated and one would anticipate a reduction of NO activity. Renal tissue was not stored from these experiments, however urinary NOx was measured and found to be ~45% lower in L257-treated animals although this did not quite reach statistical significance (p=0.07; Figure 53A).

Of note, urinary creatinine increased >2-fold which again, did not quite reach statistical significance, however no difference in plasma creatinine was observed between vehicle and L257 treatment suggesting that tubular secretion of creatinine was enhanced (p=0.09; Figure 53B).
Figure 53. Urinary NOx and creatinine

(A) Urinary NOx was reduced in L257-treated animals. Vehicle vs L257, 0.57 vs 0.32 mM (95% CIs 0.26-0.87 and 0.12-0.52; p=0.07). (B) Urinary creatinine was increased following L257 treatment. Vehicle vs L257, 30.4 vs 69.9 mM (95% CIs 24.4-36.5 and 20.6-119; p=0.09). Plasma NOx and creatinine were no different between treatments suggesting that these differences were specific to renal tubular function rather than transfer of corresponding concentrations from plasma to ultrafiltrate. Mann Whitney U test. n=5.

7.4 The effect of PTD1KO upon tubular reabsorption

Transgenic PTD1KO mice were bred as previously discussed and exported to Georgetown University for this series of micropuncture studies. As before, PT-specific DDAH1 deletion was activated using testosterone pellets (22 mg) with 90-day release, implanted subcutaneously into the mid-scapular region. After a minimum of 10 days’ testosterone treatment the mice were placed into a metabolic cages for 24hr urine collection. Microperfusion/recollection experiments were performed between days 11 and 17 post testosterone treatment.

Figure 54. The effect of PTD1KO upon Jv, urine output and sodium excretion

(A) No effect of PTD1KO was observed upon Jv; KC- vs KC+, 1.7 (± 0.21) vs 2.0 (± 0.15) nl/min/mm. (B) 24 hr urine output did not differ between groups; 1.1 (± 0.14) vs 1.2 (± 0.13) mL/24hrs and similarly, (C) sodium excretion was unchanged; 0.19 (± 0.03) vs 0.17 (± 0.02) mmol/day. Mann Whitney U test. n=6.

PT-specific DDAH1 knockdown did not affect fluid reabsorption. Similarly, 24-hour urine output and urinary sodium excretion did not differ between groups (Figure 54).
7.5 Discussion

**Key findings**

- Tubular fluid reabsorption (Jv):
  - Is significantly reduced by tubular ADMA and L-NAME perfusion
  - Is significantly reduced by systemic DDAH1 gene silencing
  - Is significantly reduced by systemic administration of DDAH1 inhibitor (L257)
  - Was not significantly altered in PTD1KO mice

Microperfusion techniques record real-time effects of local (tubular) and systemic pharmacological intervention *in vivo* and thus provide functional insights that cannot be gained using *in vitro* or *ex vivo* approaches. Tubular fluid reabsorption (Jv) was reduced by tubular perfusion of ADMA and systemic pharmacological and genetic DDAH1 inhibition. The effects of NO upon tubular reabsorption have been studied previously using microperfusion techniques and appear to show a bimodal effect dependent upon dose (see Introduction Chapter 1.7.2: Functional roles of NO in the kidney).

### 7.5.1 DDAH1 activity regulates tubular sodium handling

These data provide the first evidence that DDAH1 activity and ADMA play a regulatory role over tubular function and in these studies, ADMA inhibits fluid reabsorption, indicating that NO is stimulatory. Given that functional polymorphisms of DDAH1 are known to exist in the general population [116, 154, 217], renal sodium handling may well be influenced by DDAH1 genotype. Indeed, this is consistent with recent data from the GenSalt study; an ambitious study involving 7-day feeding of consecutive low sodium; high sodium and high potassium diets to over 3000 Chinese Han recruits with measurement of systemic BP responses and analysis of genotyping data [218]. A DDAH1 variant (rs11161637) was associated with reduced BP salt sensitivity, in that individuals homozygous for this genotype did not display the same drop in systolic BP on the low sodium diet than those with a different DDAH1 variant [219]. This confirms that DDAH1 genotypes can influence salt...
sensitivity and therefore systemic BP. What this study does not provide is data on corresponding circulating ADMA, which would shed light as to whether this effect manifests from changes in the vasculature or discrete organ beds, such as the kidney.

7.5.2 Limitations

Although microperfusion allows assessment of tubular function in vivo, the perfusate is artificial (ATF; artificial tubular fluid) and therefore its composition inevitably differs from that of ultrafiltrate. If the fluid temperature, flow, pH or tonicity is significantly different, this may have profound effects upon tubular function – more so if circulating small proteins and hormones are considered.

7.5.2.1 Tubular concentrations of NO and MAs

What these studies do not allow is the determination of NO or methylarginine concentrations within the tubular lumen as collections are of ATF, not free flow ultrafiltrate. NO concentrations within tubular fluid have been measured real-time in rats and remain in the region of 110 nM along the length of the nephron [220].

No studies to date have reported measurement of methylarginines in tubular fluid. In the L257 study, ADMA concentrations in ATF were in the region of 10 nM and assuming no contamination had occurred, this can only have existed as a result of tubular secretion of ADMA into luminal fluid. Therefore, considering normal plasma concentrations, the approximate concentration range of ADMA in tubular fluid lies between 10 and 250 nM.

Another factor, which may alter this proposed range further, is that of DDAH1 activity. In order to clarify this, tubular free flow collections would be necessary from mice treated with systemic L257; deficient in the DDAH1 gene globally and finally the PTD1KOs. Only when true tubular ADMA concentrations are known, can conclusions be drawn on the effects of physiological shifts in NO and ADMA upon tubular function.

Results from microperfusion studies in the PTD1KO mouse failed to demonstrate an effect of genotype upon sodium and fluid reabsorption. This may represent the importance DDAH1 activity within renal cell types other
than PT cells and their influence over tubular reabsorption. In addition, the presence of high L257 tubular concentrations alone may overwhelm tubular CAT transporter and hence, reabsorptive capacity. Direct measurement of ADMA within the ATF collected in these experiments would be helpful to compare against those from ADMA treatment plus free-flow collections from untreated mice.
8 MODELS OF RENAL DISEASE: CHARACTERISATION OF FOLATE NEPHROPATHY

8.1 Introduction

8.1.1 Mouse models of CKD

A variety of mouse disease models have been developed to mimic acute (AKI) and chronic (CKD) human kidney disease. Experimental approaches to invoke a renal injury fall into three broad categories: surgical, nephrotoxic and transgenic (Table 12). Whilst the specific mechanism of injury is perhaps more relevant for modelling human AKI, CKD models require simply that the final common pathway of progressive renal fibrosis is reliably established. With this in mind, the most relevant primary end-point for a model of CKD is histological assessment of tubulointerstitial fibrosis over more than one time-point. Important collateral end-points include histological evidence of macrophage recruitment, capillary rarefaction with activation of profibrotic molecules (eg, TGFβ and αSMA), elevations in plasma urea and creatinine, proteinuria and ideally, systemic BP.

Significant limitations exist with the use of mouse CKD models, both on practical terms and in reference to their application to human disease. For example, many mouse strains (particularly C57BL6) are resistant to modes of renal injury that otherwise function very well in other species such as the rat. Examples include streptozotocin-induced diabetic nephropathy and 5/6 nephrectomy [221]. In addition, many common models of CKD involve recovery surgery, requiring appropriate resource and a degree of surgical expertise to ensure reproducible results.

CKD in humans is often multifactorial and progresses over decades; features that are impractical to reproduce in an experimental setting. In addition, biological differences between mice and humans impact upon the natural history of CV and renal disease progression. As an example, wild-type mice have much higher levels of protective high-density lipoprotein (HDL),
<table>
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<tr>
<th>Model</th>
<th>Benefits</th>
<th>Limitations</th>
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<tr>
<td>Surgical</td>
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<tr>
<td>UUO</td>
<td>Reproducible histological fibrosis reported in most strains</td>
<td>Rapid progression not reflective of human disease</td>
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<td>Normal contralateral kidney compensates</td>
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<td>UNx</td>
<td>Relative ease of surgery</td>
<td>Often requires second injury (salt or protein-loading)</td>
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<td>Variable fibrotic outcomes</td>
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<td>5/6 nephrectomy</td>
<td>Well-characterised historically (esp. role of RAS in fibrosis)</td>
<td>Requires recovery surgery</td>
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<td>Disease severity varies between strains</td>
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<td>Small amount of kidney tissue to analyse</td>
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<tr>
<td>Ischaemia-reperfusion</td>
<td>Mimics a common human renal injury</td>
<td>Requires recovery surgery</td>
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<tr>
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<td>Variable fibrotic outcomes</td>
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<tr>
<td>Nephrotoxic</td>
<td>Surgery not required, two kidneys to analyse</td>
<td>Less well established and characterised than the surgical models</td>
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<td>Adriamycin</td>
<td>Proteinuric model</td>
<td>Reported success in Balb/c strain, some resistance in other strains</td>
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<td>Nephrotoxic nephritis</td>
<td>Mimics a specific human disease</td>
<td>Well established in rabbits/rats, less so in mice</td>
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<td>Highly variable disease severity, antiserum not commercially available</td>
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<td>Technically difficult tail vein injection required</td>
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<td>Streptozotocin diabetic model</td>
<td>Hyperglycaemia is predictable</td>
<td>Renal fibrosis modest and variable</td>
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<td>Strain dependent (eg. CD1 and eNOS -/- susceptible)</td>
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<td>Folic acid; adenine; aristocholic acid; cisplatin</td>
<td>Ease of administration; IP</td>
<td>Specific nephrotoxicity less applicable to common causes of human renal injury</td>
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<td>Transgenic</td>
<td>Eg. Col4α3 deletion; autosomal polycystic disease mutation; lupus-nephritis prone</td>
<td>Lifelong exposure may provoke adaption in alternative pathways</td>
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<td>Interpretation is disease-specific</td>
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<td>Costly technology</td>
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<td>Often requires second injury</td>
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**Table 12. Mouse models of renal injury**

UUO, unilateral ureteric obstruction; UNx, uninephrectomy; RAS, renin-angiotensin system; 5/6 nephrectomy, fraction of renal mass removed – unilateral nephrectomy with two-thirds of the contralateral kidney removed; IP, intra-peritoneal; Col4α3, collagen-IV subunit. (Adapted from text [221]).
which may in part explain the resistance to atherosclerosis frequently reported in mice [222].

8.1.2 Folate

Folate (folic acid or pteroylglutamic acid) is an essential cofactor required for the synthesis of pyridines and pyrimidines to create DNA. Freely filtered at the glomerulus, folate is actively reabsorbed in the proximal tubule by specialised transport proteins (α-folate receptor proteins, α-FRs) [223]. Supplementary oral folate is routinely prescribed for patients with ESRD on haemodialysis, malnourished patients, and pregnant women.

Administration of intraperitoneal (IP) folate is a well-established experimental technique to model renal injury (See Methods Chapter 3.5.8.1) [173, 175, 224]. When folate is administered in supra-therapeutic doses (180-240µg/g; ~15,000-fold higher than the 5mg daily prescribed dose in haemodialysis patients), folate crystals precipitate within renal tubule lumina and induce dose-dependent nephrotoxicity. Widespread acute tubular necrosis ensues within 48 hours with a concomitant rise in serum creatinine and BUN to mimic AKI. A period of epithelial regeneration follows, and from 2 weeks following injury, the kidneys exhibit progressive fibrosis and loss of function. Folate injury thus provides a reproducible model with which to examine the natural history of inflammation from initiating acute tubular injury through to slowly progressive fibrosis characteristic of chronic kidney disease.

Prior to adopting folate nephropathy as the model for both AKI (DDAH2) and CKD (PTD1KO) studies, it was necessary to characterise the course of folate-induced renal disease and assess the quality of data that could be gained using a variety of analysis techniques.

8.2 Study design

Forty, 10-week old female WT C57BL6 littermate mice were purchased from Charles River Ltd (Kent, UK) and randomly assigned to receiving a single IP injection of either vehicle (0.3M NaHCO₃) or folate (240µg/g, dissolved in 0.3M NaHCO₃). Mice were sacrificed at 2 days, 2 weeks and 12 weeks. A detailed description of tissue harvesting can be found in the Methods chapter.
Group sizes were between 5-8 (no premature deaths were experienced in this study).

8.3 Results

8.3.1 Kidney: body weight ratio and appearance

The kidney: body weight ratio changed appreciably over the course of the disease: initially increasing by 30% at day 2 (<0.0001) with a pale, swollen appearance (Figure 55A), followed by a return to baseline weight at 2 weeks (Figure 55B). At 12 weeks following folate injection, the kidneys had shrunk to 30% less than vehicle-treated controls with a pale, shrivelled appearance (Figure 55C).

![Figure 55. Kidney: body weight and appearance in folate nephropathy](image)

Photographs of harvested kidneys (left-sided) following vehicle vs folate administration at (A) 2 days; 6.2 vs 8.4 mg/g (95% CIs 5.3-7.2 and 7.7-9.1); p<0.0001. (B) 2 weeks; both 6.2 mg/g. (C) 12 weeks; 6 vs 4.1 mg/g (95% CIs 5.6-6.4 and 3.7-4.4); p<0.0001. Three kidneys imaged to represent; vehicle-treated n=5; folate-treated n=8. (D) Graphical representation of kidney: body weight ratios (milligrams per gram, mg/g). Two-way ANOVA with Bonferroni’s post test. n=5 (vehicle), n=8 (folate) ***p<0.0001.
8.3.2 Serum and urinalysis

At 2 days following folate administration, serum creatinine rose significantly (16-fold; p<0.001) over vehicle-treated animals, falling to 3-fold higher at 2 weeks (p<0.05) and remained 2-fold higher than controls at 12 weeks (p<0.05) (Figure 56).

![Graph showing serum creatinine, urine output, and proteinuria over time](image)

**Figure 56.** Serum creatinine (A), urine output (B) and proteinuria (C) in folate nephropathy

Serum creatinine was quantified by LC-MS/MS. (A) Serum creatinine (vehicle vs folate) at 2 days; 17 vs 276 µM (95% CIs 11-24 and 249-302); p<0.001. 2 weeks; 24 vs 65 µM (16-31 and 43-87); p<0.05. 12 weeks; 19 vs 34 µM (15-24 and 20-47); p<0.05. (B) Urine output was elevated at 2 weeks; 1.8 vs 9.9 mL/g/hr (1.2-2.5 and 6.5-13.3); p<0.001. (C) Proteinuria was raised at 2 weeks and fell by 12 weeks (not statistically significant between treatment groups). Two-way ANOVA with Bonferroni’s post test. n=5 (vehicle), n=8 (folate). *p<0.05 ***p<0.001.

For humane reasons, housing mice in metabolic cages was avoided during the period of acute inflammation hence urinary data at 2 days are not available. Mice treated with folate displayed significant polyuria at 2 weeks (5-fold; p<0.001), which reduced to a level marginally above vehicle-treated controls at 12 weeks (non-significant). Proteinuria, reported as urinary protein-creatinine ratio did not significantly change at either time point.
8.3.3 Acute inflammatory cytokines

Pro-inflammatory and pro-fibrotic cytokine mRNA transcription was up-regulated in whole kidney lysates with specific patterns over the three time-points (Figure 57). The most acute responses were exhibited by ET-1 and IL6, with transcription peaks at 2 days (5-fold and 25-fold respectively; p<0.001) before falling towards baseline at 2 and 12 weeks. TNFα mRNA transcription was delayed in comparison, with no change at 2 days and a 7-fold peak at 2 weeks (p<0.001) with a reduced but significant elevation persisting at 12 weeks (p<0.01). Housekeeper Pol2a transcription did not significantly change at any time point.

8.3.4 Pro-fibrotic cytokines

Pro-fibrotic cytokines; TGFβ and Col1 2α were up-regulated acutely at 2 days but peaked at 2 weeks (3- and 12-fold respectively) and mRNA transcription remained significantly elevated at 12 weeks (Figure 57D-E).

8.3.5 MAME expression, MAs and NO activity

Kidney tissue mRNA transcription of DDAH2 and AGXT2 was altered during the course of folate-induced kidney disease (Figure 58). DDAH1 transcription was reduced at 2 and 12 weeks although this was not statistically significant. DDAH2 transcription did not change until 2 weeks with ~3-fold upregulation (p<0.001), persisting at 12 weeks (p<0.01). AGXT2 transcription fell 37-fold at 2 days following folate in comparison to vehicle treatment (p<0.001), recovering to baseline by 12 weeks.
Figure 57. Whole kidney tissue mRNA transcription of pro-inflammatory (A-C) and pro-fibrotic (D, E) cytokines in folate nephropathy

mRNA transcription measured by RT-qPCR. Vehicle vs folate: (A) ET-1 (endothelin-1); 2 days 0.5 vs 4.6 AU (95% CIs -0.09-1.1 and 3.3-5.9); p<0.001. 2 weeks 0.4 vs 2 AU (0.3-0.5 and 0.8-3.2); p<0.05. 12 weeks 0.4 vs 0.9 AU (0.3-0.5 and 0.7-1.1); p<0.001. (B) IL6 (interleukin-6); 2 days 0.8 vs 20 AU (-0.7-2.4 and 8.9-31.8); p<0.001. 2 weeks 0.4 vs 3.2 AU (-0.02-0.8 and 1.6-4.8); p<0.01. 12 weeks 0.2 vs 3.2 AU (0.1-0.4 and -1.8-8.2); p>0.05. (C) TNFα (tumour necrosis factor-α); 2 weeks 0.3 vs 2.1 AU (0.1-0.4 and 1.3-2.9); p<0.001. 12 weeks 0.7 vs 1.6 AU (0.4-0.9 and 0.9-2.3); p<0.01. (D) TGFβ (transforming growth factor-β); 2 weeks 0.7 vs 2.2 AU (0.5-0.9 and 1.4-3.0); p<0.01. 12 weeks 0.4 vs 0.8 AU (0.3-0.6 and 0.6-1.0); p<0.05. (E) Col12α (Collagen1 subunit 2α); 2 days 0.4 vs 1.2 AU (-0.3-1.1 and 0.7-1.6); p<0.05. 2 weeks 0.2 vs 2.4 AU (0.1-0.2 and 1.1-3.7); p<0.01. 12 weeks 0.1 vs 0.6 AU (0.08-0.1 and 0.3-0.9); p<0.01. (F) Housekeeper Po2α transcription did not change. Two-way ANOVA with Bonferroni’s post test. n=5 (vehicle), n=8 (folate). *p<0.05, **p<0.01, ***p<0.001.
mRNA transcription measured by RT-qPCR. (A) DDAH1 transcription did not change significantly although there was a trend towards downregulation at 2 and 12 weeks following folate. (B) DDAH2, 2 weeks 0.3 vs 0.8 AU (95% CIs 0.2-0.4 and 0.5-1.2); p<0.001. 12 weeks 0.3 vs 0.6 AU (0.2-0.3 and 0.4-0.7); p<0.01. (C) AGXT2, 2 days 14.6 vs 0.4 AU (4.8-24.3 and 0.3-0.5); p<0.001. 2 weeks 14.7 vs 6.7 AU (10.6-18.7 and 1.4-12.0); p<0.05. 12 weeks, non-significant. Two-way ANOVA with Bonferroni's post test. n=5 (vehicle), n=8 (folate). *p<0.05 **p<0.01 ***p<0.001.

In response to folate renal injury, methylarginine concentrations in plasma, urine and whole kidney tissue changed over time (Figure 59). At 2 days, plasma L-NMMA and SDMA rose significantly in response to folate administration (8- and 4-fold respectively; p<0.001) and quickly fell towards baseline at 2 and 12 weeks. ADMA plasma concentrations did not alter at 2 days and although trended towards a rise at 2 and 12 weeks, did not increase significantly.

In urine, L-NMMA was elevated 2.5-fold (p<0.05) in folate-treated mice compared to vehicle controls but normalised at 12 weeks. Both urinary ADMA and SDMA were reduced in response to folate at 2 and 12 weeks (p<0.05).

Whole kidney content of all MAs increased by 1.5 to 2-fold at 2 days and 2 weeks (p<0.05-0.001) and fell towards baseline at 12 weeks, at which
point the only MA being significantly elevated was ADMA (approximately 25%; p<0.001).

Figure 59. Methylarginine (MA) content in plasma, urine and whole kidney in folate nephropathy

MAs measured by LC/MSMS. (A) Plasma L-NMMA and (C) SDMA were elevated at 2 days. L-NMMA (vehicle vs folate), 418 vs 3356 AU (95% CIs 72-764 and 2566-4145); p<0.001. SDMA, 4551 vs 18290 AU (1925-22753 and 13310-23270); p<0.001. (D) Urinary L-NMMA at 2 weeks, 209 vs 488 AU (138-280 and 266-710); p<0.05. (E) Urinary ADMA at 2 weeks, 80415 vs 53466 AU (61529-99301 and 42868-64065); p<0.01. (F) Urinary SDMA at 2 weeks, 36500 vs 23530 AU (26990-46010 and 16860-30199); p<0.05, similar effect remained at 12 weeks. (G) Kidney L-NMMA was elevated at 2 days 690 vs 1707 (392-988 and 1371-2043); p<0.001 and fell to non-significantly elevated levels at 2 and 12 weeks. (H) Kidney ADMA was elevated at every time-point with a peak at 2 weeks, 5978 vs 11258 AU (3856-8100 and 9837-12679); p<0.001. (I) Kidney SDMA was most elevated at 2 days, 321 vs 557 AU (200-442 and 323-791); p<0.05 and fell to control levels by 12 weeks. Two-way ANOVA with Bonferroni's post test. n=5 (vehicle), n=8 (folate). *p<0.05, **p<0.01, ***p<0.001.
Figure 60. NO activity in whole kidney and urine in folate nephropathy

NOx (nitrates and nitrites; stable end-products of NO activity) were measured by ozone chemiluminescence. (A) Kidney tissue NOx was unchanged at 2 days but rose significantly at 2 weeks, 0.006 vs 0.02 AU (0.0006-0.01 and 0.009-0.03); p<0.05. (B) Urinary NOx initially fell at 2 weeks but was increased at 12 weeks; 2 weeks 515 vs 204 AU (416-615 and 134-273); p<0.001. 12 weeks 282 vs 800 AU (241-323 and 387-1213); p<0.05. Two-way ANOVA with Bonferroni's post test. n=5 (vehicle), n=8 (folate). *p<0.05, **p<0.01, ***p<0.001.

8.3.6 Histological features

Severe tubulointerstitial disease was evident in kidney sections from folate-treated mice (Figure 61). At 2 days; tubular dilatation and epithelial flattening, luminal casts; tubular atrophy. At 2 weeks; interstitial inflammatory cell infiltrates and 12 weeks; tubular atrophy and areas of extra-cellular matrix deposition. Vehicle-treated mice displayed normal histological appearances at all time points.
Figure 61. Representative histological features of folate nephropathy

A, C and E: vehicle controls displayed normal histological appearances; dense with tubules, their lumina barely visible. B, D and F: folate-treated mice at 2 days, 2 and 12 weeks respectively. Gross and diffuse tubular dilatation (td) with some luminal casts (lc) was visible at 2 days (B). Some resolution of tubular dilatation was apparent at 2 weeks (D), but with added presence of interstitial inflammatory cells (inc) (darker nuclei). By 12 weeks (F), some earlier features remained but with marked expansion of extra-cellular matrix (ecm), tubular atrophy and some glomerulosclerosis seen lower left quadrant. Representative of n=5 (PBS vehicle) and n=8 (folate). PAS stain, x20 magnification. Bars = 50 µm.
8.3.7 Renal fibrosis (collagen deposition)

Collagen deposition quantified by picrosirius red immunostaining was significantly elevated in animals treated with folate at 2 and 12 weeks (vehicle vs folate: 2 weeks, 4 vs 5.7%, p<0.01; 12 weeks, 2.7 vs 6.4%, p<0.001. Figure 62).

8.4 Discussion

Key findings

Effects of a single IP folate injection
- Increases kidney weight by 30% at 2 days
- Decreases kidney weight by 30% at 12 weeks
- Causes significant polyuria at 2 weeks
- Increases serum creatinine 16-fold at 2 days; 3-fold at 2 weeks; 2-fold at 12 weeks
- Causes dysregulation of renal tissue methylarginine and NOx
- Upregulates kidney tissue acute inflammatory cytokine expression (ET-1, IL-6, TNFα)
- Upregulates kidney tissue pro-fibrotic cytokine expression (TGFβ, Col12α)
- Causes marked acute tubulointerstitial inflammation from 2 days to 12 weeks
- Causes significant renal tubulointerstitial fibrosis from 2 to 12 weeks

8.4.1 Folate nephropathy as a model for established CKD

These results are consistent with previous reports that folate nephropathy is a valid and reproducible model of CKD [173, 175, 224]. Renal fibrosis is the best structural correlate and predictor of renal function decline [225], proving it be the most relevant outcome in CKD models. Many published animal studies of CKD report fibrosis at a single early time-point (eg. 7-14 days post injury) which is arguably too acute, reveals nothing about disease progression and thus limits their interpretation in the context of human CKD.

In this study, significant degrees of progressive renal fibrosis were detected across two different time-points (2 and 12 weeks). Secondary outcome data supported that established CKD had been achieved by confirming; raised serum creatinine (measured by LCMS/MS), reduced kidney size, pro-fibrotic cytokine upregulation (TGFβ and Col12α) and tubulointerstitial histological changes. Furthermore, at 2 day and 2 week time-points, a sharp rise in serum creatinine, inflammatory cytokine expression and
Figure 62. Renal collagen deposition in folate nephropathy

Representational images of kidney sections from mice treated with (i-ii) Vehicle and (iii-iv) Folate. Stained with picrosirius red and viewed through circularly polarised light. A detailed description of this method, developed with the assistance of Dr Dorman (MRC CSC, London), is reported in the Methods section. Section area defined through texture thresholding (i and iii). Capsular collagen was excluded from the analysis by automated circumferential reduction of the analysed perimeter (double lines). Background is subtracted to measure percentage collagen per total section area (ii and iv). (G) Percentage collagen deposition is significantly elevated at 2 and 12 weeks. 2 weeks (vehicle vs folate); 4.0 vs 5.7% (95% CIs 3.0-5.1 and 4.4-6.9); *p<0.05. 12 weeks; 2.7 vs 6.4% (1.7-3.7 and 5.2-7.6); **p<0.01. Two-way ANOVA with Bonferroni’s post test. n=5 (vehicle), n=8 (folate). *p<0.05, **p<0.01, ***p<0.001.
acute histological changes demonstrated that folate nephropathy can also be useful to model tubulointerstitial AKI.

8.4.2 Quantifying renal fibrosis

An additional method developed during this validation study was the quantification of renal fibrosis. A commonly used approach is picro-sirius red or Masson-trichrome staining of kidney sections followed by semi-quantitative histological evaluation of collagen content and therefore fibrosis severity. This is observer-dependent, introduces potential bias, and does not take into account regional differences in collagen content across a kidney section which can vary by up to 30% [221]. The method used in this study circumvents these limitations through automated quantification of collagen as a percentage proportion of the whole section area. A potential criticism is that this quantification method inevitably includes non-fibrotic collagen of the renal pelvis structures. However, this occurs consistently within each kidney section and would simply increase the overall scores of percentage fibrosis without introducing bias for relative comparisons.

8.4.3 The NO signalling pathway in folate nephropathy

AGXT2 expression is specific to the renal tubule and is the only MAME to metabolise SDMA as well as ADMA and L-NMMA. At 2 days, renal AGXT2 mRNA transcription was profoundly suppressed by folate injury (37-fold) whilst in parallel, plasma SDMA rose 4-fold (and L-NMMA). In contrast, both DDAH isoforms and plasma ADMA were unchanged at 2 days. It is unclear whether this represents AGXT2 and SDMA as regulators or merely bystanders of tubular injury, but the actions of AGXT2 and SDMA remain largely unresolved and further study of their roles in acute renal inflammation is warranted.

There is a general perception that CKD represents a state of NO deficiency, although much of this is based upon decreased arginine synthesis and raised circulating ADMA [44]. Some of the findings here support this notion, with AGXT2 suppression and elevated kidney and plasma MAAs at 2 and 12 weeks.
In contrast however, in this study using a folate model of nephropathy, elevated NOx concentrations in kidney tissue and urine suggest that labelling CKD as a state of NO deficiency may be inaccurate. Higher renal expression of inflammatory and pro-fibrotic cytokines was observed, along with enhanced DDAH2 transcription at 12-weeks. This likely represents macrophage recruitment and activation within inflamed kidney. Taken together, these observations indicate an ADMA-NO imbalance in favour of NO predominates – not a state of deficiency.

The disparity between this study and previous reports may be explained by a difference of emphasis placed upon local kidney versus systemic NO bioavailability. Previously published conclusions that describe a state of NO deficiency in chronic renal disease are based not only upon low circulating NO and high ADMA, but also the deleterious effects of systemic administration of NOS inhibitors such as L-NAME. The vasoconstrictive effect of systemic NOS inhibition is well established, however these studies do not isolate the (extra-vascular) kidney tissue when making assessments of, or manipulating the NO-ADMA axis. This thesis and specifically, the next chapter aim to address this issue.

8.4.4 Limitations

Despite reproducibility and ease of use, the folate nephropathy model of CKD has limitations. The most immediate is the mechanism of injury, specific to the action of highly concentrated folate within the tubular lumen, which does not directly represent a specific human disease. However, CKD results when a final common pathway of progressive fibrosis is established, irrespective of the initiating injury. Therefore, perhaps the limitations of applicability to human disease are only relevant when considering its use as a model of AKI.

Although many secondary end-points were measured to confirm established CKD, proteinuria did not feature and in fact, was reduced in folate treated mice. These differences were not statistically significant and it is more likely that proteinuria detection was limited by the practical issues surrounding urine collection - as have been discussed as a significant limitation in previous chapters.
A deficiency of this particular experiment is the omission of invasive BP recordings, which would have been a useful additional outcome measure of progressive CKD and related CV disease. However, this was performed in the subsequent folate nephropathy experiment using PTD1KO mice, demonstrating a 14mmHg increase in the systolic BP of control mice (testosterone naïve; Figure 69).
9 FOLATE NEPHROPATHY IN THE PTD1KO MOUSE

9.1 Introduction

As previously discussed in Chapter 1.7.3.2, data previously published from our own group questioned the notion that raised ADMA always correlates with ill-health and is directly pathogenic. In human cohorts, a DDAH1 polymorphism (rs17384213 GG genotype) associated with higher DDAH1 mRNA expression in kidney allografts and in two independent CKD patient cohorts, lower plasma ADMA levels but a steeper rate of eGFR decline [154]. These results were in contrast to preceding association studies that indicated elevated plasma ADMA causes progressive renal disease. We took a basic science approach to resolve this conflict by specifically disrupting the NO-ADMA-DDAH axis within kidney tissue and not systemically.

The proximal tubule (PT) is the principal site of renal DDAH1 expression (Chapter 1.7.1.1). To determine the role of PT-specific DDAH1 in the progression of renal fibrosis, PTD1KO and control (KC-) mice were subjected to a folate nephropathy model of CKD.

9.2 Study design

All mice were homozygous for the floxed DDAH1 transgene but either negative or positive for the KAPICre gene (KC- or KC+). As previously described, exogenous testosterone was administered for a minimum of ten days prior to study inclusion to activate KC and delete PT DDAH1. In order to control for the effect of testosterone treatment alone, three experimental groups were necessary:

1) No testosterone, KAPICre + (T-KC+)
2) Testosterone, KAPICre - (T+KC-)
3) Testosterone, KAPICre + (T+KC+; PTD1KO)

Mice received either folate or vehicle treatment and were culled at one of three time points: 2 days, 2 weeks and 12 weeks (Table 13).
Table 13. Numbers of mice within each experimental group

Three groups; T-KC+; T-KC-; T+KC+, received either vehicle or folate and were culled at three time-points, resulting in 18 groups (122 mice total). 16 premature deaths occurred (in brackets); 8 in testosterone naïve mice treated with folate likely due to severe AKI within 72 hours (post-mortem revealed grossly enlarged, yellow kidneys); 8 testosterone-treated mice were sacrificed early after developing UV-prolapse. One mouse (T+KC+ group) was excluded from analysis due to severe bilateral hydronephrosis identified at tissue harvest. Potential effects of mortality rates are discussed (Chapter 9.4.3.1).

The KC- group size was limited by lower breeding yields of this genotype. Fewer numbers were allocated to the vehicle-treated cohorts as little or no pathological response was expected, whereas folate-treated group sizes were expanded due to a higher anticipated degree of disease variability.

9.3 Results

9.3.1 Kidney : body weight ratio

At 2 days following folate administration, kidney : body weight in T-KC+ mice had increased by 43%, whilst all testosterone-treated mice (T+KC- and T+KC+) exhibited a non-significant (variable) rise with no difference between them (Figure 63A). At 2 and 12 weeks, kidney : body weight was significantly lower in folate-treated testosterone naïve mice (T-KC+) mice compared with both testosterone treated mouse groups (T+KC- and T+KC+). Although trends for lower kidney : body weight ratios were observed in T+KC- mice compared with PTD1KOs (T+KC+), these did not reach statistical significance (Figure 63B and C).
between T
12 weeks, urinary volumes in folate vehicle controls (p<0.001 unpaired t test, not shown on graph). At both 2 and 9.3.3
fold could display signs of distress 48
Urine was not collected at the 2
9.3.2
Serum creatinine
At 2 days following folate treatment, T-KC+ mice exposed to folate (p<0.01 and p<0.001 respectively).

Serum creatinine had risen 40% following folate but this was not statistically significant. T+KC+ (PTD1KO) mouse serum creatinine did not change.

At 12 weeks, T+KC- folate-treated mice, exhibited a mean 120% elevation in serum creatinine compared to vehicle controls and T-KC+ and T+KC+ mice exposed to folate (p<0.01 and p<0.001 respectively).

9.3.3 Urinary volume
Urine was not collected at the 2-day time-point for humane reasons (mice could display signs of distress 48 hours after folate injury). At 2 weeks, a 4-fold higher urinary volume occurred in folate-treated T-KC+ compared to vehicle controls (p<0.001 unpaired t test, not shown on graph). At both 2 and 12 weeks, urinary volumes in folate-treated mice did not differ significantly between T-KC+, T+KC- and T+KC+ groups (Figure 65).

Figure 63. Kidney to body weight ratios following folate treatment
Comparison of folate-treated groups (black bars). (A) 2 days. No significant differences. (B) 2 weeks. T-KC+ vs T+KC--; 5.4 vs 8.2 mg/g (95% Cls 4.5-6.5 and 7.4-8.9); p<0.01 and T+KC+; 9.6 mg/g (95% Cl 8.4-10.9); p<0.001. T+KC- vs T+KC+; no significant difference. (C) 12 weeks. T-KC+ vs T+KC--; 4.7 vs 7.8 mg/g (4.2-5.1 and 7.8-7.7); p<0.001 and T+KC+ 8.6 mg/g (95% Cl 7.5-9.6); p<0.001. T+KC- vs T+KC+; no significant difference. Numbers per group (n): Vehicle-treated. T-KC+ 2 days n=3; 2 weeks n=6; 12 weeks n=5. T+KC- 2 days n=2; 2 weeks n=3; 12 weeks n=4. T+KC+ 2 days n=3; 2 weeks n=3; 12 weeks n=7. Folate-treated: T-KC+ 2 days n=5; 2 weeks n=4; 12 weeks n=10. T+KC- 2 days n=3; 2 weeks n=6; 12 weeks n=9. T+KC+ 2 days n=10; 2 weeks n=10; 12 weeks n=12. Two-way ANOVA with Bonferroni’s post test. **p<0.01 ***p<0.001.

9.3.2 Serum creatinine
At 2 days following folate treatment, T-KC+ mice had an 8-fold rise (p<0.001) in serum creatinine over vehicle-treated controls and both testosterone-treated groups (T+KC- and T+KC+; Figure 64A).

At 2 weeks, folate-treated T-KC+ serum creatinine had fallen but remained 60% higher than vehicle-treated animals. T+KC- mouse mean creatinine had risen 40% following folate but this was not statistically significant. T+KC+ (PTD1KO) mouse serum creatinine did not change.

At 12 weeks, T+KC- folate-treated mice, exhibited a mean 120% elevation in serum creatinine compared to vehicle controls and T-KC+ and T+KC+ mice exposed to folate (p<0.01 and p<0.001 respectively).
Comparison of folate-treated groups (black bars). (A) 2 days. T-KC+ vs T-KC-; 170 vs 13.4 µM (95% CI 126-214 and 2-24.7); p<0.001 and T+KC+ 16.7 µM (95% CI 13.3-20.2). T+KC- vs T+KC+ groups; no significant difference. (B) 2 weeks. A trend for increased serum creatinine in all folate treated animals vs vehicle controls but no significant differences. (C) 12 weeks. Higher serum creatinine in folate-treated T+KC- mice; 24.4 µM (95% CI 16.9-32) compared to folate-treated T-KC+ mice; 16.5 µM (95% CI 12.5-20.5; p<0.01), and T+KC+ mice; 12.9 µM (95% CI 11.3-14.6; p<0.001). Numbers per group (n): Vehicle-treated. T-KC+ 2 days n=3; 2 weeks n=6; 12 weeks n=5. T+KC- 2 days n=2; 2 weeks n=3; 12 weeks n=4. T+KC+ 2 days n=3; 2 weeks n=3; 12 weeks n=7. Folate-treated; T-KC+ 2 days n=5; 2 weeks n=4; 12 weeks n=10. T+KC- 2 days n=3; 2 weeks n=6; 12 weeks n=9. T+KC+ 2 weeks n=10; 2 weeks n=10; 12 weeks n=12. Two-way ANOVA with Bonferroni’s post test. **p<0.01 ***p<0.001.

(A) 2 weeks. A significant increase in urinary volume in testosterone naive mice after folate. T-KC-; 32.9 vs 125.8 µL/g/24hrs (95% CI 21-45 and 95-157); p<0.001. (A and B) 2 and 12 weeks. No significant differences in urinary volumes were detected in between folate-treated groups. Numbers per group (n): Vehicle-treated. T-KC+ 2 weeks n=6; 12 weeks n=5. T+KC- 2 weeks n=3; 12 weeks n=4. T+KC+ 2 weeks n=3; 12 weeks n=7. Folate-treated; T-KC+ 2 weeks n=4; 12 weeks n=10. T+KC- 2 weeks n=6; 12 weeks n=9. T+KC+ 2 weeks n=10; 12 weeks n=12. Two-way ANOVA with Bonferroni’s post test. ***p<0.001.
9.3.4 Proteinuria

The most significant effect upon 12-week proteinuria, was that of testosterone treatment which increased levels 4-fold (p<0.001) over controls (Figure 66). Folate produced a 2-fold (but insignificant) rise in proteinuria in T-KC+ mice but no changes in testosterone-treated groups. Although a trend of reduced proteinuria was observed in PTD1KOs (T+KC+) compared to T-KC+ mice, this did not reach statistical significance.

9.3.5 Acute inflammatory cytokines

Folate injury upregulated kidney mRNA transcription of ET-1, IL-6 and TNFα, with a peak effect between 2 days and 2 weeks (Figure 67). The largest effect was seen in T-KC+ mice (approx. 10-fold increase; p<0.01) whilst testosterone-treated groups appeared to be protected with smaller increases in cytokine mRNA between 2 and 5-fold (non-significant). At 12 weeks, overall ET-1 and IL6 transcription following folate in PTD1KOs (T+KC+) was lower than T+KC- mice although this did not reach statistical significance.

![Figure 66. Proteinuric response to testosterone and folate treatment.](image)

Testosterone had the largest impact upon proteinuria. Comparison of vehicle-treated groups (white bars). Urine protein : creatinine ratio (UPCR, mg/µM). T-KC+ vs T+KC- and T+KC+; 0.79 vs 3.6 and 3.3 mg/µM respectively (95% CIs 0.02-1.6, 1.9-5.3 and 2.4-4.2) both p<0.01. Comparison of folate-treated groups (black bars); no significant differences. **Numbers per group (n)**: Vehicle-treated; T-KC+ n=5; T+KC- n=4; T+KC+ n=7. Folate-treated; T-KC+ n=10; T+KC- n=9; T+KC+ n=12. Two-way ANOVA with Bonferroni’s post test. **p<0.01.
Figure 67. Kidney tissue acute inflammatory cytokine mRNA transcription following folate treatment.

mRNA transcription measured by qPCR. In testosterone naïve mice (T-KC+); endothelin-1 (ET-1, A-C) peak transcription at 2 days (vehicle vs folate); 4.1 vs 41.9 AU (95% CIs 1.8-6.4 and 24.5-59.3); p<0.01). Interleukin-6 (IL-6, D-F) peak transcription at 2 weeks; 0.14 vs 1.3 AU (95% CIs 0.08-0.2 and 0.89-1.65); p<0.0001. Tumour necrosis factor-α (TNFα, G-I) peak transcription at 2 weeks; 0.13 vs 1.4 AU (0.08-0.19 and 0.22-2.5); p<0.01. In T+KC- and T+KC+ groups, although trends of increased transcription following folate were observed, no significant differences were detected. **Numbers per group (n): Vehicle-treated. T-KC+ 2 days n=3; 2 weeks n=6; 12 weeks n=5. T+KC- 2 days n=2; 2 weeks n=3; 12 weeks n=4. T+KC+ 2 days n=3; 2 weeks n=3; 12 weeks n=7. Folate-treated; T-KC+ 2 days n=5; 2 weeks n=4; 12 weeks n=10. T+KC- 2 days n=3; 2 weeks n=6; 12 weeks n=9. T+KC+ 2 days n=10; 2 weeks n=10; 12 weeks n=12. Two-way ANOVA with Bonferroni’s post test. *p<0.05, **p<0.01, ***p<0.001.
9.3.6 Pro-fibrotic cytokines

TGFβ and Col12α mRNA transcription was stimulated in folate-treated mice with a peak effect at 2 weeks (Figure 68). Pro-fibrotic cytokines were most elevated in testosterone naïve (T-KC+) mice (TGFβ, 3-fold and Col12α, 14-fold) whilst testosterone treated mice had a smaller, non-significant rise.

By 12 weeks, no significant differences in TGFβ were observed. Col12α transcription however, was significantly increased by folate treatment in T-KC+ mice (>2-fold) and to a lesser extent, in T+KC- mice. PTD1KOs (T+KC+) however, exhibited significantly less Col12α kidney tissue transcription than T-KC+ and T+KC- counterparts.

![Bar graphs](image)

**Figure 68.** Pro-fibrotic cytokine mRNA transcription following folate treatment.

mRNA transcription measured by qPCR. In testosterone naïve mice (T-KC+); transforming growth factor-β (TGFβ, A-C) peak expression was at 2 weeks (vehicle vs folate); 0.4 vs 1.2 AU (95% CIs 0.33-0.44 and 0.83-1.51); p<0.001. Collagen 1 subunit 2α (Col12α; E) peak expression at 2 weeks; 0.05 vs 0.7 AU (95% CIs 0.03-0.06 and 0.45-0.96); p<0.001. (F) At 12 weeks Col12α was increased 2-fold in T-KC+ (0.4 vs 1.0 AU (95% CIs 0.23-0.64 and 0.75-1.3); p<0.01). At 12 weeks following folate treatment, Col12α transcription was significantly lower in PTD1KOs (T+KC+; 0.39 AU, 95% CI 0.27-0.52) than in both T-KC+ (1.0 AU, 0.75-1.3 AU; p<0.001) and T+KC- mice (0.78 AU, 95% CI 0.49-1.1; p<0.05). Numbers per group (n): Vehicle-treated: T-KC+ 2 days n=3; 2 weeks n=6; 12 weeks n=5. T+KC- 2 days n=2; 2 weeks n=3; 12 weeks n=4. T+KC+ 2 days n=3; 2 weeks n=3; 12 weeks n=7. Folate-treated: T-KC+ 2 days n=5; 2 weeks n=4; 12 weeks n=10. T+KC- 2 days n=3; 2 weeks n=6; 12 weeks n=9. T+KC+ 2 days n=10; 2 weeks n=10; 12 weeks n=12. Two-way ANOVA with Bonferroni’s post test *p<0.05 **p<0.01 ***p<0.001.
9.3.7 Haemodynamics

Terminal systemic BP measurements were performed in all mice of the 12-week cohort (Figure 69). At 12 weeks following folate, testosterone naïve mice had increased systolic BPs over their vehicle-treated controls (110 vs 96mmHg, p<0.05). After more than 12 weeks of testosterone exposure, T+KC- mice had elevated systolic BPs (~112mmHg) irrespective of folate treatment. PTD1KO mice (T+KC+) treated with vehicle exhibited similar increased systolic BP (107mmHg). Folate treated PTD1KO mice had significantly lower systolic BPs than T+KC- controls (98 mmHg vs 112, p<0.01).

![Figure 69. Systolic BP responses at 12 weeks following folate treatment](image)

Folate treatment increased systolic BP significantly at 12 weeks. T-KC+ vehicle vs folate; 96 vs 110 mmHg (95% CIs 90-102 and 103-117); p<0.05. Testosterone exposure increased systolic BP at 12 weeks; T-KC+ vs T+KC- vehicle-treated; 96 vs 113 mmHg (95% CIs 90-102 and 93-133); p<0.05. 12 weeks after folate, PTD1KO mice had significantly lower systolic BP than T+KC- controls; 98 vs 112 mmHg (95% CIs 93-103 and 101-122); p<0.01. Numbers per group (n): Vehicle-treated; T-KC+ n=5; T+KC- n=4; T+KC+ n=7. Folate-treated; T-KC+ n=10; T+KC- n=9; T+KC+ n=12. Two-way ANOVA with Bonferroni’s post test *p<0.05 **p<0.01 ***p<0.001.

9.3.8 Histological evidence of disease

Histological analysis confirmed significant acute tubular injury two days following folate administration in all mice. Tubular lumen dilatation, casts and epithelial cell flattening were evident from the 2-day into 2-week time-points, at which time significant interstitial inflammatory cell infiltration was also observed. By 12 weeks, areas of tubular atrophy and fibrosis were clearly seen (Figure 70).
There were no histological differences between DDAH1 intact (KC-) and PT-specific DDAH1 deleted (KC+ PTD1KO) mice treated with vehicle (0.3M NaHCO$_3$) at any time-point. At 2 days and 2 weeks following a single IP folate injection, significant disease was evident including; tubular dilatation (td) and epithelial cell flattening; luminal casts (lc) and more significantly at 2 weeks; tubulointerstitial inflammatory cell infiltrates (inc). There was no obvious difference in disease severity between KC- and KC+ mice at these time-points. At 12 weeks following folate-induced disease, tubulointerstitial fibrosis /extra-cellular matrix (ecm) deposition was more severe in KC- mice (quantified and confirmed by automated collagen analysis described in the Methods chapter and following sub-chapter).
Figure 71. Renal collagen deposition following folate treatment

Percentage (%) renal collagen deposition was assessed by automated image analysis of whole sagittal kidney sections stained with picro-sirius red (detailed in “Methods” chapter). (A) 2 days: no change. (B) 2 weeks (vehicle vs folate); T-KC+, 3 vs 6.8% (95% CIs 2.1-3.9 and 3-10.5); p<0.01. T+KC-, 2.1 vs 4.2% (0.6-3.5 and 2.6-5.8); p=0.055. T+KC+, 2.3 vs 4% (0.3-4.2 and 2.8-5.3); not significant. (C) 12 weeks (vehicle vs folate); T-KC+, 3.4 vs 7.9% (95% CIs 2.3-4.5 and 6.3-9.6); p<0.01. T+KC-, 3.8 vs 7.2% (95% CIs 2.6-5 and 5.3-9.2); p<0.05. T+KC+, 3.2 vs 4.5% (2-4.4 and 3-5.9); not significant. (D) The same data presented to highlight comparisons between T+KC- and T+KC+ (PTD1KO) mice. Numbers per group (n): Vehicle-treated. T-KC+ 2 days n=3; 2 weeks n=6; 12 weeks n=5. T+KC- 2 days n=2; 2 weeks n=3; 12 weeks n=4. T+KC+ 2 days n=3; 2 weeks n=3; 12 weeks n=7. Folate-treated: T-KC+ 2 days n=5; 2 weeks n=10; 2 weeks n=6; 12 weeks n=9. T+KC+ 2 days n=10; 2 weeks n=10; 12 weeks n=12. Two-way ANOVA with Bonferroni’s post test *p<0.05 **p<0.01 ***p<0.001.
Figure 72. Reduced collagen deposition in PTD1KO mice at 12 weeks following folate T+KC+ (PTD1KO). Representative images of kidney sections stained with picro-sirius red and viewed through circularly polarised light. Images taken at x25 magnification and processed using Image J software to stitch, remove background and quantify collagen (yellow) as a percentage of total kidney section area. (n=3 representative of; T+KC- n=9 and T+KC+ n=12 experiment group numbers).

9.3.9 Renal fibrosis (collagen deposition)

Renal fibrosis, determined by percentage collagen deposition, did not change in any treatment group at the 2-day time-point (Figure 71A). At 2 weeks, folate-treated T-KC+ mice had a significant >2-fold rise in fibrosis (p<0.01) whereas both T+KC- and T+KC+ groups had a smaller, statistically non-significant rise (Figure 71B).

At 12 weeks following folate, percentage collagen had increased further in both T-KC+ and T+KC- mouse groups but not in PTD1KOs (Figures 71 and 72).
9.4 Discussion

**Key findings**

Effects of PTD1KO at 12 weeks following folate

- Protects against serum creatinine rise
- Reduces proteinuria
- Reduces systolic BP
- Reduces pro-fibrotic cytokine response
- Prevents fibrosis

9.4.1 PTD1KO protects against progression of renal disease

Chronic kidney disease (CKD) features progressive renal fibrosis that disrupts and contracts normal kidney architecture to eventually reduce organ size and function. Clinically, CKD is defined and classified according to serum creatinine and often manifests with proteinuria and elevated systemic BP.

In this 12-week model of CKD, the PTD1KO mouse was protected from renal fibrosis; a reduction in renal mass; elevated serum creatinine; proteinuria and raised systemic BP. In addition, pro-fibrotic cytokine mRNA transcription in kidney tissue was significantly lower in PTD1KO mice than controls. These data are mutually supportive and indicate that in a folate model of CKD, DDAH1 disruption in the proximal tubule protects against kidney disease progression.

9.4.2 Conflict with previous studies: “DDAH1 protects against renal fibrosis”

Previously, a number of investigators have attempted to examine the impact of manipulating the NO-ADMA-DDAH axis in rodent models of CKD, and conclude that DDAH1 activity protects against renal fibrosis. For example, direct osmotic pump infusion of ADMA in mice exacerbated renal fibrosis
although this finding was accompanied by a 60mmHg elevation of systolic BP over controls and a similar effect with L-NAME infusion, together suggesting a predominant hypertensive injury [157].

More convincing, were studies using rodents that overexpressed DDAH1. Protection against renal collagen deposition was reported in both an angiotensin-induced and surgical nephron-reduction model of CKD (despite correcting for hypertension pharmacologically [158, 159]). Whilst intriguing, major limitations compromise their relevance to endogenous DDAH1 activity in human disease. DDAH1 overexpression was not tissue-specific and presumably included immune cells such as macrophages that do not normally express DDAH1, but play a crucial role in tissue inflammation [102]. In addition, genetic or viral over-expression of DDAH1 activity to supra-physiological levels will not be subject to normal regulatory and adaptive responses. Fundamentally, the renal-specific NO-ADMA-DDAH axis in these animals is not characterised and cannot be separated from the systemic effects of DDAH1 overexpression and BP reduction.

In the PTD1KO mouse, highly PT cell-specific DDAH1 deletion was confirmed with no alteration to baseline plasma ADMA and systolic BP, suggesting that the protection against kidney collagen deposition and functional decline was independent of circulating ADMA and BP – both intrinsic confounders in previous models. Possible mechanisms behind the influence of local DDAH/ADMA upon kidney fibrosis are discussed in Chapter 12.5.

9.4.3 Limitations

9.4.3.1 The effects of testosterone

Due to the extended nature of this disease model, mice were exposed to testosterone for longer periods of time than those in initial PTD1KO characterisation studies. By comparing data from vehicle-treated mice across all three study groups (T-KC+; T+KC-; T+KC+), physiological effects of exogenous testosterone were identified and included; increased kidney : body weight ratio (~50%, p<0.001); elevated proteinuria (4-fold, p<0.01) and raised systolic BP (17 mmHg, p<0.05). Mice exposed to testosterone appeared to
have partial protection from folate-induced acute kidney injury manifested by only very small increases in serum creatinine at the 2-week time-point; along with induced inflammatory cytokine expression and even fibrosis.

Renal hypertrophy and proteinuria associated with testosterone have been reported elsewhere [190, 226]. In a rodent model of ischaemia-reperfusion kidney injury, exogenous testosterone protected against reduced cortical perfusion and upregulated TNFα [227]. In this study, despite being initially protected from disease, testosterone-treated animals manifested similar degrees of fibrosis to testosterone naïve mice at 12 weeks. This may represent an element of hypertensive renal injury in testosterone-treated mice. In addition, testosterone has been reported to increase renal injury through exacerbating oxidative stress and pro-fibrotic angiotensinogen expression [228].

The significant effects of testosterone in this model cannot be overlooked and were to some extent, anticipated - demonstrated by the study design including two control groups. Importantly, the protective effect of PTD1 deletion was identified through comparison of two groups exposed to testosterone; KC- and KC+. Unfortunately, the very nature of this transgenic model does not allow complete separation of the effects of testosterone treatment in mice with different genotypes.

An alternative PT-specific Cre expressing transgenic mouse has been developed using a tamoxifen-responsive element driven by a promoter fragment of the (PT-specific) gamma-glutamyl transpeptidase type II gene. However, since tamoxifen is an oestrogen receptor antagonist and oestrogens are reported to reduce circulating ADMA [229], this construct would not be free of confounding effects.

Below, further specific considerations of the confounding effects of exogenous testosterone are discussed.

9.4.3.2 Mortality and testosterone effects

The fatalities attributed to AKI or acute folate toxicity (8 in total) with 72 hours of folate administration all occurred within the testosterone untreated (T-KC+) group indicating a protective effect of androgen treatment against folate injury
and disease. Apart from one mouse (1 of 53) in the PTD1KO (T+KC+) group, all other fatalities resulted from deliberate humane sacrifice due to the emergence of utero-vaginal (UV) prolapse, a known side-effect of testosterone treatment in rodents [190] (T+KC- group 1 of 28 (4%); T+KC+ 7 of 53 (13%)).

Despite a disproportionate manifestation of UV prolapse in the PTD1KO group, it is not possible to conclude as to which way this may have influenced the finding of reduced renal fibrosis in PTD1KOs (ie. producing a type 1 or narrowly avoiding a type 2 error). If testosterone protects against folate nephropathy and the incidence of UV prolapse represents testosterone sensitivity, then one could propose that the PTD1KO mouse may have incurred more protection against fibrosis over their T+KC- counterparts. However, a theory of increased testosterone sensitivity in PTD1KOs is not consistent with respect to androgen-related systemic hypertension, as PTD1KOs had lower mean systemic BP.

9.4.3.3 Serum creatinine and testosterone effects

At 2 days following folate administration, serum creatinine was significantly elevated in testosterone naïve mice but not in testosterone-treated mouse groups (T+KC- and T+KC+). Taken in isolation, this could be interpreted as a failure to produce significant acute renal injury in testosterone-treated mice. In conflict with this is the acute histological changes witnessed in kidney sections taken from testosterone-treated mice at 2 days and 2 weeks. Furthermore, at 12 weeks the presence of significant fibrosis in folate-treated mice in comparison to vehicle-treated controls indicates a folate-induced injury. In addition, albeit to a lesser extent than testosterone-naïve mice, a consistent elevation of serum creatinine concentrations at subsequent time intervals (2 and 12 weeks) and furthermore, other secondary end-points reflected much the same pattern; acute increase and subsequent decrease in kidney : body weight ratios; along with increased acute cytokine transcription (including ET-1, IL6 and TNFα).

Serum creatinine has been reported as an imperfect measure of renal dysfunction in rodent models [230] and given the complexity of effects of sex
hormones upon renal function [231], it is difficult to predict its influence upon tubular creatinine handling in the presence of additional physiological stressors. Testosterone may well protect against nephrotoxic injury, which will not only affect the degree of disease but also the natural history, meaning that 2 days may not be the appropriate time interval at which to see the most intense disease. Alternative approaches to confront or circumnavigate these issues would include using alternative measures of renal dysfunction (such as sinistrin and PAH method) and perhaps, plotting the natural history of renal injury in the presence of testosterone-treatment (eg. daily time-points within the first week).

9.4.3.4 Proteinuria and testosterone effects

PTD1KO mice had lower mean proteinuria and systemic BP than their folate-treated KC- controls. However, closer examination of the level of proteinuria and systolic hypertension in both KC- groups (vehicle and folate-treated) and the vehicle-treated group of PTD1KOs reveals comparable results. This indicates that testosterone directly elevates proteinuria and systolic BP at 12 weeks and masks the added effect of folate nephropathy. Similar to mechanisms of human CV disease, it is most likely that proteinuria develops in testosterone-treated mice as a direct result of hypertension. What is difficult to resolve is reduced systolic BP in PTD1KO only in the presence of folate injury. Although the mouse was protected against many other features of CKD, the lower BP in these circumstances may represent significant tubular dysfunction with salt and water losses significant enough to produce relative hypotension. This unmasking of tubular dysfunction with concurrent physiological stress may represent what was anticipated in the salt feeding experiments of Chapter 6.

9.4.3.5 Off-target effects of Cre activity

Deserving of comment is the possibility of Cre toxicity leading to reduced fibrosis in PTD1KO mice. Although the presence of the KC transgene was controlled for in the testosterone untreated group (T-KC+), activated KC was not, due to limitations in time and resource as this would have required a
fourth experimental group; testosterone-treated, KC+ but DDAH1 flox negative. Cre toxicity has been reported to cause cardiomyopathy [232], brain development defects [233] and widespread apoptosis in embryos [234]. To date, there are no descriptions of Cre-toxicity relating to fibrosis and any reported effects appear to disrupt normal cellular function and architecture rather than preserve it, as witnessed in the PTD1KO mouse.

9.4.3.6 Further experiments

Given the emphasis made throughout this thesis upon the differentiation of local versus systemic DDAH1/ADMA effects, an important experiment to perform would a model of folate nephropathy in global DDAH1 KO mice. Alternatively, to circumnavigate potential confounding effects of life-long genetic DDAH1 abrogation, systemic DDAH1 inhibition through administration of L257 or silencing RNA could be performed.

Data from alternative experimental models of CKD would strengthen the findings made in this study. Folate nephropathy, like many other animal disease models, does not directly represent a form of common human renal disease and its relevance to the clinical setting relies on it reaching an established “final common pathway” of fibrosis. Using a nephron reduction model of disease such as 5/6 nephrectomy, UUO model or even renal ischaemia-reperfusion to induce chronic renal disease and demonstrate protection against renal fibrosis in PTD1KO mice would inevitably add weight to the impact of this study.
10  **IN VITRO STUDIES**

10.1 Introduction

*In vivo* studies in Chapters 4, 5 and 9 indicated that increased tubular ADMA resulting from PT-specific DDAH1 gene disruption protects against the development of fibrosis following a folate model of kidney injury. An *in vitro* series of studies were undertaken to define specific effects of ADMA upon proximal tubular function.

The direct effects of folate upon PT cell function *in vitro* were not assessed for a number of reasons. The use of folate injury in this CKD model is to trigger an acute inflammatory response that transitions into a phase of chronic, progressive fibrosis to mimic the “final common pathway” observed in human CKD. It is not an examination of the long-term pathophysiological effects of (single dose) folate on the kidney. The use of folate *in vitro* would not mimic *in vivo* effects due to the absence of many stimulated cytokines and contribution of other structures and cell types such as peritubular capillaries, glomeruli and infiltrating inflammatory cells including macrophages and neutrophils. Within the limitations of *in vitro* studies, the aim here was to define the pathophysiological effects of ADMA, either directly or through pharmacological DDAH1 inhibition, upon PT cell function.

In response to injury, surviving proximal tubular cells undergo phases of differentiation and proliferation necessary for repair and restoration of function, although what determines coordinated repair from destructive proliferation is poorly understood [235]. A remarkable proliferative capacity and high metabolic strain imposed by active reabsorption, places huge energy demands upon the PT cell, which is met by densely packed mitochondria within the cell cytoplasm. Taking an *in vitro* approach, the impact of ADMA upon proximal tubular differentiation, proliferation and mitochondrial function were investigated.
10.2 Study Design

A cell line was used for the majority of these functional studies. Normal Human Proximal Straight Tubular Cells (NHPSTs) were a kind gift from Professor Patricia Wilson (UCL, London). The cells were conditionally immortalized using a vector containing pZiptsU19 with the temperature sensitive SV40 T-antigen allele tsA58U19 and a neomycin resistance gene. Proliferative growth at 33°C was switched off by incubation at 37°C within 48 hours (upon T-antigen de-activation), after which point the cells display normal PTC characteristics [160].

To support findings made in the NHPST cell line, a method for extraction and culture of primary mouse PT cells was learnt and developed. In addition, extraction of tubular fragments proved useful to:

1. confirm that renal DDAH1 expression is principally tubular.
2. demonstrate tubule-specific gene manipulation in both KAPiCre transgenic strains (ROSAyFP reporter and PTD1KO; see Results Chapter 4).

The method protocol was very kindly taught and demonstrated by Dr Linghong Huang in Dr Tim Johnson’s laboratory at Sheffield University (previously published, [236]). The technique yields purified fractions of tubular fragments that can be used for primary cell culture or immediate analysis.

10.3 Renal tubule isolation

10.3.1 Methodology

Mice were sacrificed by cervical dislocation and through a large midline laparotomy incision, the visceral fat and small bowel were moved aside to expose the abdominal aorta. Working under dissection microscopy, the renal arterial circulation was isolated by suture ligation superior to the renal arteries with additional sutures to occlude the superior mesenteric artery (SMA) and celiac trunk (Figure 73).

The abdominal aorta was cleaned of encapsulating fat and fascia and a length of suture positioned horizontally underneath the aorta (inferior to the renal arteries) and left untied. A small oblique incision was made through the
aorta just above the iliac bifurcation to approximately half the vessel width. The aorta was cannulated through this incision, using a length of pre-fashioned plastic tubing (the end was briefly heated under a light source and stretched prior to being cut obliquely to achieve a sharpened tip). The tubing within the aorta was tied in place using the pre-positioned suture. The free end of tubing was cannulated by a 30 gauge needle and attached to a 10mL syringe containing BSA-inactivated magnetic beads suspended in HBSS.

Using a syringe driver, the bead solution was infused at a rate of 1mL/min and kidney perfusion was confirmed by rapid blanching.

**Figure 73. Renal tubule isolation**

**A**, Cartoon of abdominal vessel anatomy with suture and cannulation sites indicated (adapted from Google images). **B**, microscopic examination of purified tubular fraction and **C**, glomerular fraction following magnetic separation. At higher (x400) magnification, the beads are clearly visualised within the glomerular capillary loops.

Once perfused, both kidneys were removed, decapsulated and minced with a scalpel before incubation in collagenase solution at 37°C for 30 min with agitation. The resulting homogenate was pushed through successive sieves (100µ and 50µ pore size) using a 1mL syringe plunger and ice cold HBSS rinsing. Fragments retained by the 50µ sieve were collected into a 15mL Falcon tube and a magnet was applied to the side. Sediment could be seen lining the side of the tube, representing glomeruli with magnetic beads trapped within their capillary loops (Figure 73C). The tubular fragments and cells remained in suspension (Figure 73B). Microscopic examination of tubular and glomeruli fractions confirmed purity and fractions were pelleted by
centrifugation at 1,000rpm for 5mins before resuspension in media and plated out or stored at -80°C until further use.

The most technically challenging aspect was correct placement of sutures to avoid liver and gut perfusion leading to inadequate renal bead entrapment. The SMA and celiac trunk (leading to the hepatic and splenic arteries) branches lie very close to the right renal artery especially, and care was required to tie ligatures around them whilst avoiding compromise to right kidney perfusion. All too often in early experiments, was the liver perfused causing inadequate bead perfusion into the glomerular capillary loops. Once well practiced, the surgical success rate was in the region of 95% (approximately 1 in 20 were inadequately perfused).

10.3.2 Characterisation of tubular / glomerular fractions

10.3.2.1 MAME/NOS expression

RT-qPCR and Western blotting analysis confirmed that the tubules chiefly express renal DDAH1 and AGXT2 (96% and 98% more than glomeruli; p<0.001) whilst the glomeruli express proportionately more DDAH2 (53%), iNOS (99%) and eNOS (82%; all p<0.001). nNOS was equally expressed in both fractions (Figures 74A-D). The glomerular fraction had 13-fold (p<0.01) more NOx content than the tubule.

10.3.2.2 Cell surface protein expression

Expression of E-cadherin and pancytokeratin (both epithelial (tubular) cell markers) and alpha smooth muscle actin (α-SMA; a marker of myofibroblasts and glomerular mesangial cells) were studied to assist in estimating fraction purity. The relative expression of each marker within the fraction it did not represent was between 20-28% (Figure 74F and G).
Figure 74. MAME and NOS expression profiles of isolated tubular / glomerular fractions.

All data expressed as relative percentage expression between tubular and glomerular fractions. mRNA corrected by Pol2a and protein, by α-tubulin transcription. Successful staining with antibodies for iNOS and AGXT2 protein detection was not possible despite attempts to optimise those commercially supplied (iNOS, author's own findings; AGXT2, Dr B Caplin personal communication). Mann-Whitney U test. n=5 all groups. **p<0.01, ***p<0.001.
10.4 The effects of ADMA upon proximal tubular cell function

10.4.1 Differentiation: NHPSTs

Transforming growth factor β (TGFβ) is a cytokine secreted by activated immune cells that stimulates cellular differentiation and proliferation. Its role in renal fibrosis is well recognised [237] and its use in vitro allows specific pathways of fibrosis to be studied. TGFβ has been shown to stimulate epithelial to mesenchymal (EMT) in vitro and in vivo [238, 239], and despite the controversy over which cells of origin contribute to myofibroblasts in vivo [240, 241], a state of epithelial de-differentiation / differentiation following injury is generally accepted.

By quantifying the expression of protein markers of epithelial (E-cadherin) and myofibroblast or mesangial (αSMA) cell types, the differentiating effect of TGFβ upon NHPSTs was examined and furthermore, whether treatment with ADMA (3µM; plasma concentrations observed in patients with ESRD) or DDAH1 inhibitor, L257 (100µM) altered this effect.

Treatment with 5ng/mL TGFβ produced significant phenotypic changes in NHPSTs at 96 hours with E-cadherin suppression (32%; p<0.05) and αSMA stimulation (53%; p<0.05) suggesting differentiation from an epithelial cell phenotype to mesenchymal (or myofibroblastic). No additional effect was seen with either ADMA or DDAH1 inhibitor treatment (Figure 75).
Figure 75. The effect of TGFβ upon NHPST differentiation at 96 hours

Using Western blotting analysis, a significant effect of TGFβ upon NHPST differentiation was observed with no added effect of ADMA or DDAH1 inhibitor. A and B: loss of E-cadherin expression. Control vs TGFβ; A, 0.91 vs 0.67 AU (95% CIs -0.61-2.44 and -0.19-1.53; p<0.05), B, 0.79 vs 0.48 AU (95% CIs 0.25-1.33 and 0.22-0.74; p<0.05). C and D: gain of αSMA expression. Control vs TGFβ; C, 0.94 vs 1.92 AU (95% CIs -0.49-2.38 and 1.3-2.46; p<0.05), D, 0.29 vs 0.62 AU (95% CIs 0.09-0.49 and -0.97-2.21; p>0.05). E, Representative Western blot. Concentrations used: TGFβ 5ng/mL, ADMA 3µM, DDAH1 inhibitor (L257) 100µM. Results indicative of n=3 individual experiments. 4 wells per experiment. One-way ANOVA with Bonferroni’s post test. *p<0.05, **p<0.01.

10.4.2 Proliferation: NHPSTs

Proliferation in response to serum feeding (10%) (after 48 hours of serum-starving) was measured using two methods; a scratch wound assay and Bromodeoxyuridine (BrdU) assay (see Methods). Cells treated with FCS grew
>4-fold more than those in serum free media (p<0.001; Figure 76). ADMA treatment (3 and 10µM) reduced growth by ~35% (p<0.01) and DDAH1 inhibitor L257 by 20% although this did not reach statistical significance. Mitomycin reduced growth by 63% (p<0.001). As mitomycin is a powerful inhibitor of proliferation, it suggests that growth of cells into the wound is largely proliferation rather than migration.

![Figure 76](image)

**Figure 76. The effect of ADMA and DDAH1 inhibition upon NHPST proliferation**

A. Representative images of the same scratch wound taken at the time of treatment (T0) and 24 hours later (T24) reveal cellular growth into the space. B. Representative images of treatment groups following digital track pad demarcation. The filled (black areas) were quantified using Image J software and percentage growth calculated and plotted in C. Comparisons of FCS only vs other treatments. FCS vs serum free; 48.3 vs 11.3% (95% CI 32.4-64.3 and 6.2-16.5); p<0.001. FCS vs ADMA; 3µM and 10µM both 32% (95% CIs 27.7-36.3 and 9.6-64.4 respectively); p<0.01, and mitomycin 18%; p<0.001. Results indicative of n=3 individual experiments. 4 wells per experiment. One-way ANOVA with Bonferroni’s post test. **p<0.01, ***p<0.001.

To confirm an inhibitory effect of ADMA upon NHPST proliferation, a second assay was used; a bromodeoxyuridine (BrdU) assay (Figure 77). This also provided further information in that the inhibitory response appeared to
be dose-dependent over a range of 1.0 - 10µM (p<0.001), and that the NOS-inert methylarginine isoform SDMA had no effect (suggesting that the ADMA effect was through NOS inhibition). Similar to the scratch studies, DDAH1 inhibitor L257 had a weak but statistically non-significant inhibitory effect on proliferation.

![Figure 77. BrdU assay in NHPST cells](image)

FCS only (black bar) vs FCS + other treatment comparisons. Linear trend across ADMA concentrations (horizontal bar). FCS only: 1.67 arbitrary units (AU, 95% CI 1.4-1.93) vs ADMA 1, 3 and 10µM: 1.12 (p<0.01), 0.96 (p<0.01) and 0.89 (p<0.001) respectively (95% CIs; 0.92-1.33; 0.86-1.07; 0.79-0.99); and for linear trend showing dose-dependent ADMA effects p<0.001. No significant difference with SDMA or DDAH1 inhibitor (L257) treatment. Results indicative of n=3 individual experiments. 8 wells per experiment. One-way ANOVA with Bonferroni’s multiple comparison test and post test for linear trend. **p<0.01 ***p<0.001.

10.4.3 Proliferation: primary PTCs

Studies in the NHPST cell line had confirmed an inhibitory effect of ADMA upon proliferation and it was desirable to confirm this in a culture of primary tubular cells. Renal tubules were isolated from mice (WT, C57B6 strain) as previously described and cultured in 96 well plates.

In an effort to repeat the scratch wound assays, cultures of primary cells were attempted on numerous occasions in 6, 12 and 24 well plates with no success. The unresolved issue was that of patchy growth and a failure to reliably cultivate uniform cell monolayers necessary for the technique. For this reason, an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-
(4-sulfophenyl)-2H-tetrazolium) assay was used alongside the BrdU proliferation assay used previously.

Results between the two assays were very similar; the dose-dependent inhibitory effect of ADMA upon proliferation seen in the cell line was confirmed \( (p<0.01) \) and furthermore, an inhibitory effect of DDAH1 inhibitor L257 seen only as a trend in the NHPST cell line, was found to be statistically significant \( (p<0.05) \).

Figure 78. Proliferation assays in primary tubular cells

FCS only (black bar) vs other treatment comparisons. Linear trends (horizontal bars) across ADMA concentrations. **A**, BrdU assay. FCS vs serum-free; 0.24 AU vs 0.13 (95% CI 0.2-0.29 and 0.11-0.15); \( p<0.001 \). ADMA 1, 3 and 10\( \mu \)M; 0.17, 0.16 and 0.12 (\( p<0.05 \)) AU respectively (95% CI 0.07-0.26; 0.08-0.21; 0.09-0.15); \( p<0.01 \) for linear trend showing dose-dependent ADMA effects. FCS vs L257; 0.24 vs 0.14 AU (95% CI 0.07-0.22); \( p<0.05 \). **B**, MTS assay. FCS vs serum-free; 0.83 vs 0.54 AU (95% CI 0.76-0.89 and 0.47-0.62); \( p<0.001 \). ADMA 1, 3 and 10\( \mu \)M; 0.79, 0.70 and 0.67 AU respectively (95% CI 0.7-0.88; 0.58-0.81; 0.57-0.77); \( p<0.01 \) for linear trend. FCS vs L257 0.83 vs 0.66 AU(95% CI 0.55-0.78); \( p<0.05 \). Results are indicative of \( n=4 \) individual experiments. 8 wells per experiment. One-way ANOVA with Bonferroni’s multiple comparison test and post test for linear trend. \*\( p<0.05 \) **\( p<0.01 \) ***\( p<0.001 \).

10.4.4 Mitochondrial function in NHPSTs

Proximal tubular (PT) cells are densely packed with mitochondria, allowing them to produce large quantities of ATP required to fulfil their energy-demanding reabsorptive role. NO is known to influence mitochondrial function at many levels as discussed in Introduction Chapter 1.7.2.5. Both in vitro and in vivo studies presented in this thesis demonstrate significant ADMA-dependent effects upon PT cell function.
It was hypothesised that ADMA reduces PT cell proliferation through disruption of mitochondrial respiration.

To test this, the NHPST cell line was used to assess the effect of ADMA and the synthetic NO donor (SNAP) upon mitochondrial respiration using two \textit{in vitro} approaches:

1) Measurement of real-time aerobic and anaerobic respiration using the Seahorse XF Extracellular Flux Analyzer (Seahorse Bioscience, Copenhagen, Denmark).

2) Measurement of intracellular adenosine mono-, di-, tri-phosphates by LCMSMS to calculate energy charge. NHPST cells were grown to confluence in 12-well plates and treated with ADMA (10 µM) and SNAP (100 µM). Each well was harvested in ice-cold perchloric acid (20%).

10.4.4.1 Seahorse experiments

This automated system quantifies the cellular respiration rate in basal and stressed conditions by using compounds to influence parts of the mitochondrial respiratory chain. NHPST cells were seeded to achieve confluence within 48 hours and cells were treated with ADMA 10 µM and NO donor (S-nitroso-N-acetyl-l,l-penicillamine, SNAP; 100 µM) for 24 and 48 hours.

Following ADMA treatment for 24 and 48 hours, basal oxygen consumption rate (OCR) significantly increased (~20%; p<0.05) whilst NO donor (SNAP) treatment caused a decrease (~40%; p<0.01) (Figure 79A,B,E). These effects diminished during mitochondrial stress and there were no effects of ADMA or SNAP treatment upon anaerobic glycolysis (as measured by extracellular acidification rate (ECAR); Figure 79C,D).

10.4.4.2 Energy charge

The energy charge, or status of a cell can be determined by taking into account the relative abundances of ATP, ADP and AMP using the equation [242]:

$$\text{Energy charge} = \frac{[ATP] + (1/2[ADP])}{([ATP] + ADP + AMP)}$$
The normal baseline physiological range for the energy charge of a cell is 0.8-0.95. Lower values suggest higher metabolism, whilst higher values indicate lower metabolism as less ATP / ADP is being used for energy-consuming functions. Using LCMS/MS, both ADP and AMP were optimised for reliable detection although this was not possible for ATP at the time of study (this has since been achieved). Therefore, an abbreviated version of the formula was used to determine energy charge (giving a lower normal range):

\[
\text{Energy charge} = \frac{ADP}{[ADP + AMP]}
\]

When the energy charge was quantified in NHPST cells, treatment with ADMA significantly reduced the energy charge (13%; p<0.05), whilst SNAP treatment caused an increase (74%; p<0.001) (Figure 79F).

### 10.5 Discussion

**Key findings**

**Isolation of renal tubules**
- Achieves separation of tubular cells with 80-99% purity
- Confirms that renal DDAH1 and AGXT2 expression is highly tubule-specific
- Provides viable cultures of primary tubular cells

**In a proximal tubular cell line (NHPSTs)**
- TGFβ causes trans-differentiation from an epithelial to mesenchymal phenotype
- ADMA or DDAH1 inhibition does not affect trans-differentiation
- ADMA inhibits proliferation
- DDAH1 inhibitor L257 inhibits proliferation
- ADMA increases (and NO decreases) basal mitochondrial respiration
- ADMA decreases (and NO increases) NHPST cell energy charge
Figure 79. Mitochondrial function in NHPSTs

Media and compounds (1-4) were added sequentially to modulate mitochondrial function and their effect on oxygen consumption rate (OCR) was measured. (1) control medium; (2) oligomycin to inhibit ATP synthase; (3) FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), an uncoupler to short-circuit the proton circuit and allow maximal respiration; (4) rotenone to inhibit total mitochondrial respiration. Production of lactic acid (hydrogen ions; extracellular acidification rate (ECAR)) was also measured to quantify anaerobic glycolysis. A and B, following 24 and 48 hrs of treatment, consistent trends were observed for increased basal OCR with ADMA treatment (the difference was subsequently lost following the addition of compounds 2-3). Consistently, a decrease in OCR was seen with SNAP (NO donor) treatment. Although these differences did not reach statistical significance according to two-way ANOVA testing, focussed analysis of basal OCR (*E*) revealed significant effects of ADMA (46.6 pM/min; 95% CI 40.5-53.2; p<0.05) and SNAP (23.1 pM/min; 95% CI 16.6-29.1; p<0.01) when compared to control (38.4 pM/min; 95% CI 34.9-41.9). F, in separate in vitro studies, LCMS/MS quantification of ADP and AMP in NHPST cells treated with ADMA and SNAP produced a significant decrease and increase respectively, in cellular energy charge: Control 0.39 AU (95% CI 0.35-0.43) vs ADMA 0.34 AU (95% CI 0.32-0.36; p<0.05) and SNAP 0.68 AU (95% CI 0.65-0.72; p<0.001). One-way ANOVA with Bonferroni’s post test. All n=4 (6 wells per group per experiment). *p<0.05 **p<0.01 ***p<0.001.
10.5.1 Renal tubule isolation

10.5.1.1 Tubular purity and protein characterisation

In this study, the magnetic bead perfusion technique for separating and purifying fractions of tubules was successful, achieving degrees of purity (80-99%) similar to previously published reports [236, 243]. Assessments of tubule purity were made according to relative expressions of proteins specific to epithelial (E-cadherin and pancytokeratin) or glomerular mesangial cells and myofibroblasts (αSMA). In the case of primary cell culture, the media composition positively selected epithelial cell growth thereby suppressing the growth of any non-tubular cells.

Highly tubule-specific DDAH1 and AGXT2 expression demonstrated here is not only consistent with previous reports [70, 98, 99], but suggests limited contamination of tubular fractions by other renal cell types. There remains uncertainty as to the nature and site of iNOS expression in the kidney. Some investigators have been unable to detect iNOS in untreated human, rat or mouse kidney tissue [244], whilst others have identified iNOS expression at various points along the tubule in basal conditions [71] and in the glomerulus during acute inflammation [245]. Interestingly in these studies, iNOS mRNA transcription was detected and predominant in the glomerular fraction (99%) and barely detectable in tubular fractions. This may represent glomerular disruption and induced iNOS expression resulting from mechanical trauma during the extraction process. In addition, much of the renal iNOS expression data derives from rat studies [70] [[246] and may reflect differences between species.

10.5.1.2 Proximal tubule purity

Using this method, isolated tubular fragments pertain to different regions of the entire nephron – not solely the proximal region. Attempts were made to quantify proximal tubule cells using FACS analysis (with Aquaporin 1 and Megalin antibodies) without success, largely due to difficulties in liberating single cell suspensions from tubular fragments. However, the majority of kidney mass is composed of proximal tubule and in studies using stereological histology, the estimated proportion of total kidney mass was
approximately 65% [191]. The remaining components (connective tissue, vasculature and glomeruli) are extracted through sieving and magnetic separation, which would result in proximal tubule purity far in excess of the initial 65%. In the instance of primary culture, media components positively select PT growth over other cell types thus increasing PT cell purity further still.

10.5.2 ADMA inhibits PT cell proliferation

The most prominent observation was that ADMA inhibited proliferation of NHPST and primary PT cells at concentrations comparable to those measured in the plasma of patients with CKD (1-10 µM). This is the first demonstration of ADMA inhibiting renal PT cell proliferation, however similar effects have been shown in cultured alveolar epithelial cells, in which both ADMA and a DDAH1 inhibitor (L291) inhibited proliferation and induced apoptosis [127]. The anti-proliferative effect of ADMA appears to be specific to the epithelial cell sub-type. Previously, investigators have reported that ADMA inhibits VEGF-induced motility in HUVECs but it did not alter endothelial cell proliferation [247]. In activated macrophages, ADMA inhibits migration and phagocytic function but similarly, proliferation was unaffected (Ahmetaj and Leiper, personal communication).

Renal tubular cell de-differentiation and proliferation occurs following acute kidney injury [235]. Dysregulation of these processes has been implicated in the pathogenesis of the ensuing chronic and progressive fibrosis [248]. Supporting folate nephropathy as a model that is representative of human renal disease, tubular epithelial cell de-differentiation and proliferation has been observed following experimental folate administration in mice [249, 250]. In the studies presented here, ADMA inhibits tubular cell proliferation in vitro. In mice with PT-specific DDAH1 gene disruption, tubular cell ADMA was elevated and consequently, were found to be protected from progressive renal fibrosis at 12 weeks following folate injury. Taken together, these findings indicate that tubular ADMA may confer protection against fibrosis through limiting the pathological proliferation that can ensue following acute kidney injury. Further studies, such as comparing the transcription or protein
expression of proliferating cell nuclear antigen (PCNA) in KC- and KC+ (PTD1KO) mice would be useful to confirm this.

Tubular cell apoptosis is critical to renal tubule responses in acute injury and the transition to chronic fibrosis [248]. The folate nephropathy model of renal disease has been shown to manifest tubular apoptosis in experimental mice [250, 251]. Some preliminary in vitro studies were designed to investigate the effects of ADMA in TGFβ-induced apoptosis with limited success (data not shown); largely due to inadequate caspase 3 induction and/or detection using Western blotting. Further in vitro studies would be useful to further investigate this, along with supportive data of kidney caspase induction from in vivo studies presented in Chapter 9.

10.5.3 ADMA alters mitochondrial respiration

These data demonstrate that ADMA increases OCR and decreases cellular energy charge in NHPSTs. Logically, NO donor SNAP had the opposite effect. This is consistent with the current dogma suggesting NO inhibits mitochondrial respiration by binding to cytochrome c oxidase [252].

Given that ADMA-treatment increased PT cell oxygen expenditure but reduced energy charge, an uncoupling effect of ADMA upon mitochondrial ATP generation is suggested. A previous study of hepatic mitochondrial dysfunction in diabetic rats demonstrated that ADMA caused mitochondrial dysfunction, reduced ATP with evidence of increased oxidative stress. These findings were replicated in hepatocyte in vitro experiments along with enhanced transcription of uncoupling protein 2 (UCP2) [253]. The uncoupling of mitochondrial function may underlie the effect of ADMA treatment inhibiting PT cell proliferation and potentially, explain some of the effects seen on tubular reabsorptive function, which clearly require further study to confirm.

10.5.4 Limitations

10.5.4.1 Quantifying PTC purity

Prior to adopting the magnetic bead technique, a number of different approaches for renal tubule purification were attempted, involving multiple sieving steps or Percoll centrifugation to separate fractions [254, 255].
Magnetic bead perfusion was by far the most successful, but despite almost complete separation of glomeruli from tubules, the purity of proximal segments was difficult to quantify. As discussed previously, attempts at using FACS analysis of tubular fractions failed, however an alternative approach could involve growing the PTCs out in culture first, to generate a cell monolayer from which it is easier to gain a single cell suspension. Subsequent incubation with antibodies against PTC-specific (Megalin, Aquaporin 1) and non-PT proteins (NCC and Aquaporin 2; characteristic of distal tubule and collecting duct) prior to FACS analysis would give quantitative data on purity. A consideration for this strategy however, is the phenotypic changes invoked in cells that are taken out of their normal environment altering characteristic protein expression, such as reduced megalin production for example.

10.5.4.2 Mitochondrial metabolism

Results of the investigation into effects of ADMA upon mitochondrial activity were intriguing, however they are only preliminary, requiring further study before conclusions can be drawn. A limitation to Seahorse analysis is the requirement for a uniform cell monolayer – something that can be achieved in the NHPST cell line but not in primary PTCs. This obviously precludes primary cells from being used to replicate and support effects seen in the cell line.

At the time of study, LCMS/MS detection of adenosine phosphates was not fully optimised, with ADP detection being sub-optimal and ATP detection completely absent. Although the results presented here were robust and reproducible, they require repeating now that detection of all three subtypes has subsequently been optimised. Furthermore, demonstrating a dose response relationship of ADMA and SNAP would add further strength to these findings, plus an investigation into UCP mRNA transcription (using RT-qPCR) would help to confirm or refute the hypothesis of an uncoupling effect of ADMA.
11 THE ROLE OF DDAH2 IN AKI

This project was run in parallel to, and independent of the renal DDAH1 studies.

11.1 Introduction

The second DDAH isoform (DDAH2) was identified a decade after DDAH1 [101, 256] and has been much more difficult to characterise. Data from transgenic overexpression experiments suggest that DDAH2 can have a lowering effect on circulating ADMA, however demonstration of its activity in humans has been difficult to detect. Technical difficulties in producing a soluble, active recombinant DDAH2 protein have halted enzyme characterisation studies and prevented screening of compounds that may enhance or inhibit activity. Studies investigating rodent overexpression [124] or human polymorphisms of DDAH2 [257] suggest that DDAH2 can determine plasma ADMA concentrations. Both DDAH isoforms are strongly expressed in visceral organs including the liver, kidney and pancreas but distinct expression patterns exist in other tissues, with high DDAH2 expression often co-localising with eNOS in vascular endothelium, heart, lung, placenta and most exclusively, in immune cells [102].

11.1.1 DDAH2 in macrophage activation and disease

Recent observations within our group identify DDAH2 as a critical enzyme in macrophage-driven inflammation. Both DDAH2 gene deletion and exogenous ADMA, inhibit macrophage migration and phagocytic burst activity (unpublished, Ahmetaj and Leiper), whilst global DDAH2 gene deletion appears to protect against joint inflammation in a rodent model of rheumatoid arthritis (unpublished, Leiper). To date, the effect of DDAH2 gene overexpression or deletion in inflammatory disease has not been reported although DDAH2 overexpression in mice appears to reduce ADMA-induced vascular oxidative stress and hypertension [124].

Macrophage activation and infiltration are prominent features of various experimental models of renal disease including; ischaemia [258],
glomerulonephritis [259] and folate nephropathy [224, 250]. Furthermore, studies presented in this thesis (Chapter 8.3.5) identified a 3-fold increase in kidney tissue DDAH2 transcription at 2 and 12 weeks following folate injury; which likely represents macrophage activation and infiltration.

11.1.2 Hypothesis

Given the evidence for the contribution of DDAH2 in macrophage activation and inflammatory disease, we tested the hypothesis that DDAH2 null (knock-out; KO) mice would be protected from acute renal inflammation.

11.2 Nephrotoxic nephritis model

The nephrotoxic nephritis (NTN) model of disease was originally selected to test the hypothesis. As glomerular and interstitial macrophage infiltration is a hallmark feature of the NTN model [260], it seemed an ideal method to use. The model was performed with assistance and nephrotoxic serum provided by Dr Ruth Tarzi, Imperial College, London, and is described in detail in the Methods chapter.

A pilot experiment involved 5 female DDAH2 KO mice and WT littermate controls aged between 8-12 weeks. 7 days following pre-immunisation, 5mg of nephrotoxic serum (NTS) was administered via the tail vein and mice were checked daily for 10 days until sacrifice and tissue harvesting. Mortality was higher in the WT group (3 of 5) than the DDAH2 KO group (1 of 5). Although the small remaining numbers made statistical testing impossible, histological findings were promising – revealing more severe disease in WT kidney tissue (Figure 80).
Figure 80. Representative kidney PAS-stained histology following NTN induction
(A) WT tissue revealed tubule dilatation (TD), some with luminal casts, tubular cell flattening and evidence of glomerular crescents (arrow, bottom left). (B) DDAH2 KO tissue appeared unaffected. Representative of 2 in the WT group, 4 in the DDAH2 KO group. Bars = 50 µm.

The model was repeated using 10 mice in each group and, given the unacceptable mortality experienced in the pilot experiment, the interval between pre-immunisation and NTS administration was reduced from 7 to 5 days. The second experiment caused no mortality but also failed to produce any significant disease according to histological appearances. A third experiment was performed, again using 10 mice in each group but this time using a new batch of NTS at an increased dose of 10mg (from 5mg). Yet again, this failed to produce significant disease according to histology.

At this point, the NTN model was abandoned due to such variable disease – a finding previously reported by others [221]. Results in parallel studies using folate nephropathy as a model of CKD suggested that it yielded much more reproducible disease and that it could also be used to model the acute phase of renal inflammation.

11.3 Study design

Female mice, both DDAH2-/- and WT littermates (8 - 10 weeks old) were treated with a single IP dose of folate as previously described. To illustrate the dose-sensitivity of different strains, these mice developed significant disease at a lower dose of 180 µg/g (240 µg/g in the PTD1KO strain). Mice were culled 2 days later and tissues harvested. Baseline urine and post treatment
plasma and kidney tissue were collected and stored at -80°C. The experiment was repeated four times in total with 5-12 in each treatment group.

11.4 Results

11.4.1 Baseline plasma and urine MA concentrations

Plasma MA concentrations did not differ between WT and D2KO mice at baseline whereas in urine, levels were significantly higher in D2KOs; L-NMMA >4-fold (p<0.05) and ADMA >2-fold (p<0.001; Figure 81).

**Figure 81. Plasma and urine MA concentrations**

(A) No differences in plasma MA levels were observed. (B) Urinary MA concentrations were significantly higher in D2KO mice. WT vs D2KO; L-NMMA, 0.077 vs 0.338 µM (95% CIs 0.04-0.11 and 0.15-0.52 respectively), p<0.05; ADMA, 18.8 vs 44.4 µM (95% CIs 12.7-24.9 and 37.1-51.8), p<0.001. Mann-Whitney U test, n=7 (WT) and 11 (D2KO). *p<0.05 ***p<0.001.

11.4.2 Renal gene expression

As anticipated, DDAH2 mRNA expression in kidney tissue from D2KOs was absent whereas in WT mice, it doubled at day 2 post folate treatment (p<0.01; Figure 82A). Additionally at 2 days, induced expression of acute inflammatory cytokines (iNOS, TNFα and IL1β) was significantly lower in D2KO renal tissue and measurement of urinary NOx suggested that renal NO activity was also reduced (Figure 82).
Figure 82. Renal tissue gene expression and urinary NOx; 2 days post folate

(A) DDAH2 mRNA expression was detected at levels 50-fold lower in D2KO mouse kidney tissue and deemed insignificant (95% CI 0.004-0.045AU). Folate treatment increased renal DDAH2 expression in WT mice from 0.95 to 1.94 AU (95% CIs 0.89-1 and 1.47-2.4; p<0.01). (B) urinary NOx was reduced in D2KO mice; WT vs D2KO, 1.4 vs 0.59 mM (95% CIs 0.49-3.29 and 0.33-0.84; p<0.05). Cytokine expression was also reduced with the exception of IL6; (C) iNOS, 35.1 vs 1.97 AU (95% CIs 5.8-64.3 and 1.0-2.9; p<0.001); (D) TNFα, 14.9 vs 0.48 AU (95% CIs -3.1-32.9 and 0.27-0.69; p<0.01); (E) IL1β, 2.05 vs 0.67 AU (95% CIs -1.5-5.6 and 0.46-0.88; p<0.05); (F) IL-6 transcription was not significantly different. n=7 (WT) and 11 (D2KO). WT comparisons in folate vs vehicle-treated groups (A); two-way ANOVA with Bonferroni's post test, **p<0.01. WT vs D2KO comparisons (B-F); Mann-Whitney U test. *p<0.05 **p<0.01 ***p<0.001.
11.4.3 Macrophage recruitment

Immunohistochemistry using a Mac-2 antibody revealed significantly more macrophages in WT mouse renal tissue than D2KOs at 2 days following folate administration (14 vs 5; p<0.01; Figure 83).

![Figure 83. Renal macrophage recruitment at 2 days post folate](image)

(A) WT vs D2KOs, 14.2 vs 5.2 (95% CIs 3.5-25 and 2.9-7.6; p<0.01). Representative kidney tissue images from (B) WT mice showing evidence of macrophage infiltration (arrows) with significantly lower numbers observed in renal tissue taken from D2KO mice (C). Sections stained with anti-Mac-2 antibody (Abcam). Means derived from counts within 4 randomly selected cortex fields for each section. Values verified by a second independent blinded observer. n=4 (WT), n=10 (D2KO). Mann Whitney U test. *p<0.05.

11.4.4 Plasma creatinine and mortality

Renal function according to plasma creatinine was preserved in D2KO and significantly elevated in WT mice at 2 days following folate administration (p<0.01). In the initial study, overall mortality was 43% in WT and only 9% in D2KO mice (Figure 84).
Figure 84. Plasma creatinine and mortality

(A) Plasma creatinine was significantly elevated from baseline in WT and not in D2KO mice; WT vs D2KO, 125.1 vs 26.5 µM (95% CIs 63.7-186.5 and 16.4-36.6; p<0.01). (B) In a pilot study using 240µg/g of folate, mortality was 43% compared to only 9% in D2KO n=4 (WT), n=10 (D2KO). The folate dose was reduced to 180µg/g for subsequent experiments to reduce mortality and allow the experiment to run to term (2 days). n=7 (WT) and 11 (D2KO). Mann-Whitney U test. **p<0.01.

11.5 Discussion

Key findings

DDAH2 KO mice:

- have elevated urinary methylarginines at baseline

In a folate model of AKI, DDAH2 KO mice are protected from:

- Inflammatory cytokine expression (iNOS, TNFα, IL1β)
- Renal tissue macrophage infiltration
- Elevated urinary NOx
- Raised plasma creatinine
- Mortality

In this folate model of AKI, DDAH2 null mice were protected against renal macrophage infiltration, cytokine expression and decline in renal function according to serum creatinine. Furthermore, in an initial study using a higher 240µg/g dose, mortality was 34% lower in DDAH2 deficient mice (for subsequent experiments, 180 µg/g was used).

This is the first such study to demonstrate a role of DDAH2 in renal inflammation and is consistent with previous observations made by our group in studies of macrophage function and rheumatoid arthritis. Evidence
suggests that the effect of DDAH2 is mediated through NO regulation. Through comprehensive in vitro studies of macrophages taken from WT, macrophage-specific and global DDAH2 KO mice, Dr Ahmetaj (Leiper group) demonstrated that NO synthesis was reduced in DDAH2 KO strains when stimulated with an inflammatory cocktail (LPS, TNFα, IFNγ). The addition of iNOS inhibitor 1400W almost completely abrogated nitrite production suggesting that DDAH2 activity is required for complete iNOS activation during inflammation (Figure 85).

![Nitrite production graph](image)

**Figure 85. Macrophage NO activity in response to LPS stimulation**

NO activity according to nitrite production. ddah2 +/-, WT; ddah2 m-/-, macrophage-specific DDAH2 KO; ddah2 +/-, global DDAH2 KO; 1400W, iNOS inhibitor. Comparisons between DDAH2 +/- and other genotypes. One way ANOVA, *p<0.05. Courtesy of Dr B Ahmetaj, MRC, London.

In this folate model of AKI, urinary NOx was also reduced in DDAH2 KO mice in support of the protective effect against inflammation manifesting through reduced NO synthesis.

A requirement for tonic NO activity to achieve iNOS activation has been described elsewhere [261]. Organ bath studies of rat aortic rings denuded of endothelium and hence eNOS activity, were resistant to iNOS activation (within the medial layer) in response to LPS stimulation. A similar phenomenon may explain the findings in DDAH2 KO mice. Increased ADMA in the macrophages of DDAH2 null mice would suppress basal NO production and subsequently, limit iNOS activation in response to inflammatory stimuli.
Using non-selective pharmacological NOS inhibition (L-NMMA) to reduce the deleterious effects of NO activity in human sepsis, unfortunately increased mortality in clinical trials [75]. This indicates that therapeutic non-selective NO reduction is unlikely to be of benefit and therapies are required to effect a targeted manipulation of the NO signalling pathway. DDAH2 is expressed across specific cell types and represents a target for selective pharmacological inhibition of NO production, theoretically providing clinical benefit of reduced inflammation but tolerable side effects. At present, a specific DDAH2 inhibitor is not available for use – largely due to the current technical limitations in producing a soluble recombinant DDAH2 protein required for enzyme kinetic and screening studies. Encouragingly however, selective inhibitors of DDAH1 have been developed and show therapeutic promise in protecting against circulatory collapse seen in bacterial sepsis [262]. This indicates that a selective inhibitor of the second DDAH isoform is not an unrealistic expectation.

11.5.1 Limitations

An obvious limitation to this study is the use of folate nephropathy to model AKI. Renal folate toxicity does not occur in humans and therefore the relevance of the model relies upon comparisons made between this and forms of tubulointerstitial renal disease seen in patients such as drug toxicity (cisplatin, aminoglycoside or ethylene glycol) or multiple myeloma (characterised by casts within tubular lumina causing obstruction with interstitial inflammation). Similar to folate nephropathy and these human conditions of acute tubular necrosis (ATN) are histological features including; tubular casts and dilatation with epithelial cell flattening; inflammatory cell infiltrates and disruption of normal cortical architecture. Folate nephropathy has previously been recognised as an experimental model of AKI [263] and like any other model of human disease, results have to be considered in the context of disease- and species-related limitations.
11.5.2 Further work

Further studies will be useful to confirm a protective effect of DDAH2 abrogation in inflammatory disease is a) effected through macrophages and their activation, and b) replicable in other forms of renal inflammation and species. Macrophage-specific DDAH2 KO mice have previously been created and characterised by Dr Ahmetaj within our own group (unpublished; under the LysMCre promoter [264]). Repeating a folate model of AKI or perhaps an alternative injury, such as ischaemia-reperfusion in this mouse strain would determine whether the protective effect of DDAH2 abrogation is conferred through effects on the macrophage cell type.

As discussed above, the nephrotoxic nephritis (NTN) model is highly variable in the mouse. In the rat however, NTN is an established model of crescentic glomerulonephritis and is much more reproducible [265]. Additional advantages of working with the rat in preference to the mouse exist in terms of handling, administering compounds, obtaining plasma and urine along with the relative ease in performing surgical procedures. For these reasons, creation of a global DDAH2 gene KO rat would be a valuable resource for developing the findings made in these preliminary studies.
The overall aim of this thesis was to test the hypothesis: “Reduced renal DDAH1 activity protects against kidney function decline”. To achieve this, a novel transgenic mouse was generated and characterised before subjecting it to a folate model of CKD. Supportive in vivo microperfusion and in vitro studies provided mechanistic data evaluating the direct effect of ADMA upon proximal tubular cell function.

12.1 Development of the PTD1KO mouse

A large proportion of this thesis focussed upon the generation and characterisation of a proximal-tubule specific DDAH1 knock-out mouse (PTD1KO). Given that the PT cell is the principal renal cell-type to express DDAH1 and plays key roles in CKD progression, this transgenic strain was vital in testing the original hypothesis.
Experiments in Chapter 4 demonstrate that the KC construct confers PT specificity in the YFP reporter and PTD1KO mouse. Although KC activity was detected in the liver and brain, DDAH1 expression was not significantly reduced in these organs so however doubtful their influence over fibrosis in distant organs such as the kidney, it was even less likely to confound experiment results. Consequently, tubular-specific effects of PTD1KO upon reduction of ADMA and NO synthesis were confirmed, whilst levels in plasma, urine and even whole kidney were unaffected. Such cell-specific manipulation of DDAH1 has not been reported previously and for this study, provides clear advantages over previously developed models that exhibit global gene disruption [105, 119, 266, 267].

12.1.1 Influence of gender in mouse studies

The use of only female mice may have impacted upon the outcomes in salt feeding and folate nephropathy experiments. Gender-related effects upon kidney disease have been reported in both humans and mice [268]. Female mice have previously been demonstrated to manifest significant acute and chronic renal disease in response to folate administration [175]. Female mice appear to be protected in other experimental disease models of metabolic and cardiovascular disease [269], but appear to suffer more adverse outcomes in autoimmune disease models [270] (personal communication for Dr Ruth Tarzi, Imperial College, London; mouse nephrotoxic nephritis model). In cardiovascular and renal disease, gender differences are thought to reflect the androgen-oestrogen axis [207]. For this study, it is difficult to anticipate the overall contrasting effects of exogenous testosterone in female mice, however this has to be considered when interpreting the results.

12.2 Limited biochemical effects of PTD1KO

Despite data confirming successful disruption of PT-specific NO-ADMA-DDAH axis in vivo, biochemical evidence of disrupted PT reabsorption in PTD1KOs was lacking. The effects of NO signalling over mitochondrial activity and PT reabsorptive function are well described and therefore, a phenotype was anticipated to resemble Fanconi’s syndrome; a condition of PT cell
dysfunction manifesting inadequate reabsorption of glucose, electrolytes and amino acids [271, 272]. A non-significant but reproducible trend for lower systolic BPs in PTD1KO mice was observed along with elevated urinary amino acids, glucose and lactate. This indicated a possible Fanconi-like effect, however due to the high variability in urinary data, any differences according to genotype were rendered statistically insignificant.

The limitations of urine biochemistry profiling are discussed at the end of individual chapters 5 and 6, and pertain to issues surrounding contamination of urine collected in metabolic cages. Further still, the effects of testosterone upon body weight, urinary volumes, creatinine and proteinuria confound results; altogether making small effects of PTD1KO upon tubular function impossible to confirm using these methods.

In addition, the design of some experiments in terms of time intervals may have limited observed effects. For example in the salt-feeding studies, 8 days' of salt-feeding may not be long enough to manifest physiological effects. Consistent with this, a trend for increased systemic BP was observed in the high salt groups, although this did not reach statistical significance. Further studies would be undertaken to optimise the salt exposure through higher concentrations and extended time periods beyond 2 weeks.

12.2.1 Limitations of testosterone use

As previously discussed (Chapters 5 and 9), the confounding effects of testosterone cannot be overlooked. The effects seen in these studies, including total body weight gain, kidney hypertrophy, proteinuria and after long-term exposure, increased systemic BP have all been reported with exogenous testosterone use previously [190, 205, 226]. The renal tubule is known to be hormone sensitive [185], which is an insurmountable issue that needs to be considered here in the use of KAPiCre as a means of achieving transgenic PT cell selectivity. For this reason, key comparisons to isolate the effects of DDAH1 deletion were made between two groups, both treated with testosterone (T+KC- and T+KC+).

Alternative transgenic constructs to target the PT cell are available but fail to offer significant advantages over the KAPiCre construct. A PT-specific
Cre using a tamoxifen-responsive element driven by a promoter fragment of the (PT-specific) gamma-glutamyl transpeptidase type II gene has been used [273]. Since tamoxifen is an oestrogen receptor antagonist and oestrogens are reported to reduce circulating ADMA [229], this construct would not be free of confounding effects. Others have targeted the Na+/H+ exchanger to achieve PT cell gene disruption, but this also has profound effects upon fluid and electrolyte flux across the gastrointestinal mucosa where it is also expressed [274].

12.3 Urinary proteomics – an alternative approach

Urinary profiling using a proteomic approach was much more fruitful, identifying 84 significantly altered peptides out of a total of 1437 measured. Explanations as to why this type of analysis unveiled significant differences whilst biochemical profiling did not, arise from the aforementioned issue of contaminated urine collections. Many of the peptides identified in the proteomic study are specific to plasma (albumin) or even urine (UMOD, MUPs, KAP) and not present in mouse chow, water or faeces. Their presence therefore, is likely to reflect true content as opposed to detection of contaminating salts for example – an inherent problem in the biochemical assays.

A screening approach such as this is useful for hypothesis-generation, and despite the majority of identified peptides originating from proteins of unknown or uncertain function, has identified far-reaching downstream effects of tubular DDAH1 deletion. Intriguingly, UMOD and Col1a1 were the two most significantly down-regulated peptides in PTD1KO mouse urine, which may represent particular significance in the context of progressive renal fibrosis, as discussed in more detail below.

An observation worthy of mention is the detection of urinary albumin peptide. Although clinical dogma implies that the glomerulus is impermeable to albumin, microperfusion studies in normal rats confirm quantities exist in the ultrafiltrate (20-30µg/mL) but are almost completely reabsorbed through endocytosis within the PT - hence albumin is not usually found in urine [275]. Recently, testosterone has been shown to directly increase albuminuria in
mice [205]. The two groups compared in the urinary proteomic study (KC- and KC+ or PTD1KO) were both treated with testosterone and while each had significantly elevated proteinuria over testosterone-naïve controls, PTD1KO mice had almost 6-fold less albumin peptide in the urine. Albuminuria can result from hypertensive glomerular injury as witnessed by mice exposed to testosterone for 12 weeks, however these mice were normotensive after only ~10 days of treatment, thus making a direct inhibitory effect of testosterone over PT endocytic reabsorption much more likely as the cause of urinary albumin leak. The 6-fold reduction in PTD1KO mice indicates that DDAH1 deletion in part, reverses or compensates for testosterone-dependent endocytic dysregulation, in the PT; providing further evidence that DDAH1 influences PT reabsorption.

12.4 PTD1KO mice are protected from renal fibrosis and functional decline

The severity of renal fibrosis is the most valuable predictor of functional decline in human CKD [225]. Data presented in Chapter 9 indicate that PTD1KO protects against renal fibrosis at 12 weeks following folate injury. Furthermore, secondary outcomes of disease included lower proteinuria, systolic BP, serum creatinine and renal tissue pro-fibrotic cytokine expression (TGFβ and Col12α). Together, these data form a convincing argument for PTD1KO limiting kidney fibrosis and functional decline but they do not reveal the mechanism(s) through which this occurs.

12.5 Proposed mechanisms of protection against fibrosis

12.5.1 ADMA inhibits PTC proliferation

In vitro studies presented in Chapter 10 demonstrate that ADMA inhibits PTC proliferation. Taken in isolation, this can be interpreted in either direction: that ADMA reduces normal proliferative responses to injury (and therefore harmful), or that it limits pathological, unmeasured proliferation that persists in CKD (and therefore beneficial). In the context of results from the folate nephropathy model in PTD1KO mice, one can postulate the latter to be true,
that PT DDAH1 disruption protects against destructive PT cell proliferation, deposition of interstitial collagen deposition and ultimately, preserves kidney function. Indeed, the same protective effect of pharmacological DDAH1 knock-down in a rodent model of pulmonary fibrosis has been previously reported, where proliferation of primary alveolar epithelial cells was inhibited by a selective DDAH1 inhibitor (L291) whilst rodents overexpressing DDAH1 had more severe pulmonary fibrosis following bleomycin injury [127].

Furthermore, preliminary data presented here suggests an uncoupling effect of ADMA upon mitochondrial respiration, which could underlie the suppressed proliferative capacity of PT cells and altered reabsorptive function, however this requires confirmation with further study.

12.5.2 ADMA inhibits PTC reabsorption

Microperfusion studies found that both ADMA and synthetic non-selective NOS inhibitor L-NAME, inhibited PTC sodium and water reabsorption (Jv) when perfused directly into the tubular lumen. Furthermore, DDAH1 inhibitor (L257) reduced Jv, although this was required to be infused systemically for it to have an effect. Unfortunately similar effects were not replicated in microperfusion studies using the PTD1KO mouse (discussed in Chapter 7).

If the effect of tubular ADMA is to selectively inhibit reabsorption of sodium and other molecules, this may provide the PTD1KO mouse with protection from renal fibrosis for a number of reasons. Like many ultra-filtered substances, folic acid is reabsorbed in the PT using specific folate-binding proteins expressed at the apical surface [276]. Supra-physiological concentrations of folate administered to mice in this model crystallise in the tubular lumen causing obstruction and possibly, direct cytotoxicity [277]. Inhibited reabsorption of folate and sodium (and therefore water) would thus protect PTD1KO mice from intracellular folate accumulation and even luminal crystallisation due to conservation of water within the ultrafiltrate.

By inhibiting tubular reabsorption overall, ADMA may confer protection against fibrosis by lowering metabolic demands upon the PT, with reduced free radical production and subsequently, suppressed stimulation of pro-inflammatory pathways.
Finally, ADMA inhibiting tubular reabsorption would also limit renal salt and water retention, suppress elevations in systemic BP and thus avoid a “second-hit” hypertensive renal injury.

12.6 The role of UMOD in renal fibrosis and CKD

Urinary proteomic data present alternative mechanisms of protection against renal fibrosis in PTD1KO mice. Urinary peptides Col1a1 and UMOD were lower in urine from PTD1KO mice at baseline (8.5- and 6.5-fold respectively). Collagen type 1 predominates in renal fibrosis [278]. Reduced production of collagen type 1 subunits by tubular cells and fibroblasts at baseline in PTD1KOs may be extended or even accentuated in a pro-inflammatory environment, thus conferring protection against tubulointerstitial fibrosis.

More intriguing, is the reduction of urinary UMOD in PTD1KO mice. The immunomodulatory effects of UMOD have already been discussed in Chapter 5 but in summary, studies show considerable conflict as to whether UMOD behaves as a pro- or anti-inflammatory protein. Initial observations in humans are equally contentious. Rare monogenetic UMOD-related tubular diseases are characterized by defective urinary concentrating ability, low urinary UMOD levels, progressive tubulointerstitial fibrosis with UMOD aggregates visible within TAL epithelial cells suggesting defective protein transport [195]. However a recent review of UMOD biology identifies that; low urinary UMOD is often a feature in genetic disease; either low or high concentrations are demonstrated across an impressive array of renal conditions; but in established CKD, UMOD levels are elevated [279].

Genome-wide association studies (GWAS) support this and provide insights into the significance of urinary UMOD in a general population. A number of UMOD polymorphisms have been described to associate with hypertension, CKD and kidney function [195]. A recent case control study revealed that elevated urinary UMOD preceded the development of CKD over a ten-year follow-up. In addition, they identified a protective UMOD C allele that correlated with lower urinary UMOD levels in a dose-dependent manner [280].
The correlation of reduced urinary UMOD with DDAH1 deletion in the PTD1KO mouse may represent an element of tubular dysfunction and impaired protein trafficking, although rather than pre-disposing to fibrosis as seen in UMOD-related genetic disease, it conferred protection against fibrosis in the folate experiment. These findings indicate that UMOD turnover (and perhaps TAL cell function) is strongly influenced by upstream DDAH1 activity and consistent with the GWAS data, UMOD contributes to kidney disease progression.

12.6.1 Human evidence of an association between DDAH1 and UMOD

Independent to this thesis, work undertaken by Dr Ben Caplin confirms an association between renal DDAH1 expression and urinary UMOD in a human renal transplant cohort (unpublished; UCL, London). Analysis of renal biopsy tissue and urine from recipients of live kidney transplant graft reveals a strong correlation between renal DDAH1 expression and urinary UMOD. When extended to the whole cohort (live and deceased donors), the association was rendered insignificant. This likely reflects the heterogeneity of organs from deceased donors and relatively small sample number (Figure 86).

The study described at the start of this thesis, implicating a DDAH1 gene variant that associated with higher kidney tissue mRNA expression, lower circulating ADMA, and faster rate of progressive kidney function decline was also published by Dr Ben Caplin [281]. The in vivo work presented in this thesis supports his human cohort findings and taken together, indicate that renal DDAH1 activity and UMOD expression contribute to progressive renal function decline independent of circulating ADMA.
12.6.2 Conflict with published data: does DDAH1 prevent or cause disease?

These human cohort and animal model studies are mutually supportive and suggest that renal DDAH1 activity exacerbates kidney function decline. On perfunctory examination, they stand in contrast to the wealth of human observational data indicating that elevated circulating ADMA exacerbates, whilst DDAH1 protects against, CV and renal disease [106, 108-111]. Even recently, reduced DDAH1 activity has been implicated in human disease through identification of loss-of-function DDAH1 polymorphisms that associate with elevated plasma ADMA and an increased risk of CV events [116, 117].

The critical issue to reconcile this controversy is that of tissue-specificity. The majority of studies implicating ADMA in disease do so by reporting circulating ADMA, whereas here, focus upon ADMA levels within the kidney produces contrary results. The interpretation of associations found between plasma ADMA and disease is limited; in that risk factors for CV and renal disease are themselves reported to have relationships with plasma ADMA, so defining a direction of causation is confounded [91]. Using circulating ADMA as a marker of disease across specific tissue beds can therefore be misleading, as indeed suggested by a study exposing DDAH1
overexpressing mice to angiotensin II induced hypertension, which revealed variable hypertensive injury between the heart, vasculature and kidney [282].

Given the ubiquitous nature of NO signalling, it is plausible that competitive inhibition of NOS by ADMA can be both protective as well as destructive, determined by the disease conditions according to site, chronicity and magnitude of responses. It appears that at least in pulmonary [127], and now kidney fibrosis, reduced NO availability through DDAH1 disruption and elevated ADMA, is protective.

12.6.3 Relevance to human disease

12.6.3.1 Therapeutics

Tissue-specificity, a major strength in this study, presents challenges to the direct transfer of results towards a therapy for CKD. Pharmacological DDAH1 inhibition using compound L257 shows tissue selectivity and protection against circulatory collapse in a mouse model of bacterial sepsis [262]. The same study demonstrated that L257 inhibited NO production in LPS-treated aortic tissue but not macrophages. This reflects relative expression of DDAH1 in vascular and macrophages (which do not express DDAH1), explaining the systemic BP benefit of both genetic and pharmacological DDAH1 inhibition during sepsis, whilst macrophage function is unaffected.

Unfortunately, the pattern of DDAH1 expression cannot be exploited for the treatment of CKD. Chronic administration of L257 is likely to increase systemic blood pressure or at least, alter autoregulation of organ perfusion, which would clearly have a negative impact upon renal function and progressive fibrosis. Furthermore, acute kidney injury was not obviously attenuated in PTD1KO mice suggesting that timed or pulsed DDAH1 inhibition at an early stage of renal disease is equally unlikely to hold therapeutic promise. (As discussed in Chapter 11 however, such acute disruption of the DDAH2 isoform is an intriguing possibility). Indeed, unless targeted manipulation of the NO-ADMA-DDAH axis is achieved, many therapies are likely to fail as exhibited by previous human clinical trials in peripheral vascular disease [93] and bacterial sepsis [75], in which outcomes actually worsened with intervention.
Rather than proposing a direct therapeutic intervention for progressive renal fibrosis (CKD), this thesis provides important insights into the role of DDAH1 and ADMA in disease and serves as a caution to therapies aimed at non-selective ADMA reduction. Pharmacological agents already used for alternative indications have been shown to enhance DDAH1 activity and increased ADMA clearance. Rosiglitazone, an agent for the treatment of diabetes has been reported to help reduce plasma ADMA levels [283]. Additionally, a functional promoter element of Farnesoid X receptor exists within intron 1 of DDAH1 gene. When FXR agonists are administered to rats, DDAH1 transcription increases with subsequent reduction of plasma ADMA [284]. Furthermore, administration of an FXR agonist (INT-747) was also seen to reduce plasma ADMA and enhanced insulin sensitivity in Dahl-sensitive rats [285]. These findings have led investigators to propose potential benefits of enhancing DDAH1 activity to treat diseases that feature elevated ADMA, such as CV disease. Results from this body of work indicate that such an approach could have deleterious consequences, at least in the kidney.

12.6.3.2 Risk stratification

As previously discussed, genetic variants of DDAH1 can increase the risk of CV disease, determine BP responses to dietary salt and accelerate the progression of kidney function decline in CKD. As we are only just emerging into the post-genome era, personalised medicine according to genotype and even metabolic make-up is a far-off, yet attractive possibility [286]. Knowledge of an individual’s DDAH1 genotype or intra-renal activity for example, prior to renal transplantation or at the time of diagnosing CKD, would guide supportive therapies and improve longer-term outcomes.

12.6.4 Further work

A number of observations made in this thesis deserve further study. The transgenic PTD1KO mouse represents a valuable resource for investigating the effects of NO signalling in proximal tubular cells. Although initial microperfusion studies were disappointing in terms of demonstrating an effect of PTD1KO upon sodium and fluid reabsorption, the experimental design had
certain limitations and urinary proteomic studies suggested significant changes in tubular function. Future micropuncture experiments in PTD1KO mice would be of interest and should include; analysis of free flow tubular fluid (for MAs, NOx, amino acids and electrolytes), perfusion of different ATF compositions (electrolytes, amino acids, low molecular weight proteins) or compounds to profile the features of tubular reabsorption in PTD1KO mice. Furthermore, comparison with global DDAH1−/− mice would help to separate the local versus systemic effects that have been frequently emphasised in this thesis.

The role of DDAH1 activity and ADMA in regulating PTC mitochondrial activity also represents a further avenue of investigation. Preliminary results indicate that ADMA uncouples mitochondrial respiration, which may represent the mechanism underlying inhibited PTC reabsorption or proliferation. In vitro studies using the NHPST cell line and primary PTCs from WT and PTD1KO mice would allow LCMS/MS analysis of adenosine phosphates and thus estimation of their energy charge. RT-qPCR analysis would also determine whether cellular respiration effectors such as uncoupling proteins (UCPs) are implicated in NO-dependent effects.

The association of urinary UMOD with renal DDAH1 expression is intriguing, as both are reported to associate with renal function decline. Confirmation in both the PTD1KO mouse and a human renal transplant cohort suggest that this warrants further study with regard to; direct influence of NO and ADMA upon TAL cell UMOD production and handling; confirming the same association in larger cohorts of CKD patients; defining a urinary proteomic profile that could predict kidney function decline.

12.7 Conclusion

The overall aim of this thesis was to test the hypothesis: “Reduced renal DDAH1 activity protects against kidney function decline”. Using a novel transgenic mouse with proximal tubule-specific DDAH1 deletion, protection against renal fibrosis and functional decline was demonstrated in a folate model of chronic kidney disease.
Characterization studies of the PTD1KO mouse employed a variety of experimental techniques and provided novel insights into the role of renal DDAH1 activity in health and disease. Furthermore, collaborative in vivo studies involving microperfusion, and in vitro studies provided additional insights into the role of ADMA and DDAH1 regulating NO in the proximal tubule.

This work emphasizes the importance of considering local versus systemic ADMA and DDAH1 activity, along with the potential pitfalls of accepting circulating ADMA as a marker or therapeutic target in organ-specific disease.
35. Mark MacGregor, M.T. Detection, Monitoring and Care of Patients with CKD. Renal Association Clinical Guidelines 2011.


120. Hu, X., et al., Vascular endothelial-specific dimethylarginine dimethylaminohydrolase-1-deficient mice reveal that vascular endothelium


14 PUBLICATIONS AND PRESENTATIONS RESULTING FROM WORK DURING THIS PHD

14.1 Publications

1) Submitted:


14.2 Oral presentations

14.2.1 Regional

1) Chain-Florey Clinical Research Fellowship Workshop, MRC CSC, Imperial College London, Hammersmith Hospital Campus, 3rd Oct 2013.
   “*Reduced renal DDAH1 activity protects against progressive kidney damage*”

2) Renal Forum, Hammersmith Hospital, 11th April 2013. “*The role of DDAH1 in proximal tubular cell function and response to injury*”
14.2.2 National

1) Renal Association Congress, Bournemouth, 13-15th March 2013. “DDAH2 (dimethylarginine dimethylaminohydrolase) is essential for a complete inflammatory response in a folate model of acute kidney injury”


14.3 Poster presentations

14.3.1 Regional


14.3.2 International

1) Kidney Week 2013, ASN, 5-10th Nov 2013, Atlanta, GA, USA. “Reduced renal DDAH1 activity protects against progressive kidney damage”

World Congress of Nephrology 2013: 31st May – 4th June, Hong Kong:

2) “DDAH2 and the inflammatory response in acute kidney injury”

3) “Novel in vivo techniques confirm a role for the ADMA-DDAH pathway in the regulation of proximal tubular sodium transport”

6th International Symposium on Asymmetric Dimethylarginine, 30-31st August 2012, London:

4) “DDAH2 is essential for a complete inflammatory response in a folate model of acute kidney injury”

5) “A novel investigative tool for the role of DDAH1 in the kidney: conditional proximal-tubule-specific DDAH1 deletion”
6) Kidney Week 2011, ASN, 8-13th Nov 2011, Philadelphia, USA. “Anti-proliferative effect of asymmetric dimethylarginine (ADMA) - a clue to renal fibrosis?”
## 15 APPENDIX

### LCMS/MS fragment detection data

<table>
<thead>
<tr>
<th>Compound</th>
<th>Detector mode</th>
<th>Intact m/z</th>
<th>Fragment m/z</th>
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### Primer Sequences: genotyping

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### Primer Sequences: quantitative PCR

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228
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