

**Chronic kidney disease associated cardiovascular disease: scope and limitations of animal models**

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**Abstract**

Chronic kidney disease (CKD) is a heterogeneous range of disorders affecting up to 11% of the world's population. The majority of patients with CKD die of cardiovascular disease (CVD) prior to progressing to end stage renal disease (ESRD).

CKD patients have an increased risk of atherosclerotic disease as well as a unique cardiovascular phenotype. There remains no clear aetiology for these issues and a better understanding of the psychopathology of CKD associated CVD is urgently needed.

Although non-animal studies can provide insights into the nature of disease, the whole-organism nature of CKD-associated CVD means that high quality animal models, at least for the immediate future, are likely to remain a key tool in improving our understanding in this area.

We will discuss the methods used to induce renal impairment in rodents and the methods available to assess cardiovascular phenotype and in each case describe the applicability to humans.

**Keywords:** Animal models, cardiovascular disease, chronic kidney disease, translational models

## Article

Chronic kidney disease (CKD) is the term given to a heterogeneous range of disorders affecting the structure and function of the kidney. It is a global health problem, affecting up to anywhere between 9%-11% of the world's population [1]. It is driven by a broad range of aetiologies and can progress to end-stage renal disease (ESRD), a condition which can only be treated by dialysis or renal transplantation. However, populations with CKD, pose a substantial healthcare burden aside from provision of renal replacement therapy, mainly attributable to cardiovascular (CV) morbidity and mortality. Indeed the majority of patients with CKD will die from cardiovascular disease (CVD) prior to progressing to ESRD [2].

Compared to the general population patients with CKD have a higher burden of traditional CVD risk factors. These in turn behave in a more accelerated fashion, for example, CKD patients have more severe coronary artery atherosclerotic plaque formation compared to the general population [3,4] and the risk of myocardial infarction among CKD patients is twice that of patients without CKD [5]. Similarly, peripheral artery disease [6] and stroke [7] all show increased risk as the estimated glomerular filtration rate begins to fall below 60ml/min per1.73m<sup>2</sup>.

Alongside increased risks of atherosclerotic disease CKD also leads to a distinctive cardiovascular phenotype characterised by prominent endothelial dysfunction, arterial stiffening and calcification alongside left ventricular hypertrophy, fibrosis and cardiac dysrhythmia. CKD-associated CVD typically leads to clinical outcomes such as admission for heart failure and sudden cardiac death. It is these clinical events that constitute the leading causes of morbidity and mortality in patients receiving dialysis [8]. Although a number of underlying risk factors and mechanisms for this clinical syndrome have been described including proteinuria [9], anaemia[10], salt retention [11], retention of uraemic toxins [12], inflammation [13] and oxidative stress [14] there remains no clear aetiological framework for these problems. A better understanding of the pathophysiology of CKD-associated CVD is therefore urgently required, both to better understand the nature of the risk, and

to develop novel therapies aimed at reducing the burden of CVD in this population. Although epidemiological, clinical translational and *in vitro* studies can provide insights into the nature of disease, the whole-organism nature of CVD-associated CVD means that high quality animal models, at least for the immediate future, are likely to remain a key tool in improving our understanding in this area.

Most scientists aspire to a future where use of cell-based systems and cross-platform bioinformatics approaches make animal experimentation redundant but currently rodent disease models provide a unique insight into whole organism physiology and pathophysiology. However whilst animal models of disease remain necessary all studies should be designed to minimise suffering and in accordance with the “Three R’s”. That is, “Replacement” of animals with other methods if possible, “Refinement” of animal models to maximise data gained per animal and “Reduction” of animals used where possible [15].

There is a long history of modelling chronic renal injury in rodents[16]. These models can be used to: (i) simulate specific diseases that cause CKD, for example diabetes [17] or systemic lupus erythematosus [18]; (ii) examine the final common fibrosis pathways of progressive CKD; or (iii) to investigate CKD complications such as CKD associated CVD. It is this third aim that will be the focus of this review.

Although atherosclerotic disease and the consequences of athero-occlusive events have been modelled in animals in the context of renal injury (e.g. ApoE KO [19] or coronary artery occlusion in rat models [20]), we focus on models aimed at investigating the distinctive cardiovascular phenotype of CKD associated CVD in this review. We will discuss the methods used to induce renal impairment in rodents (Table 1) and the methods available to assess cardiovascular phenotype (Table 2) and in each case describe the applicability to humans.

### **Rodent Models of CKD**

**Unilateral ureteric obstruction (UUO):** The UUO model has been performed in rats and mice. One ureter is tied off causing increased urinary tract pressure proximal to the lesion followed by an interstitial inflammatory response with subsequent cellular invasion and eventual tubulointerstitial fibrosis and atrophy. The degree of fibrosis is proportional to the length of time the ureter is tied off [21]. In general the contralateral kidney, which in situ, provides a control “normal” kidney to be analysed histologically alongside the obstructed one. The advantage of this model is that it is technically less challenging than nephrectomy, it is easily reproducible and works in most strains of both mice and rats. The renal damage occurs rapidly, reaching peak within 7 days. However, whilst human CKD can be caused by urinary obstruction [22], overall it is not a major cause of CKD in the adult population, and even when it is, partial rather than complete obstruction is typical [23]. Furthermore in rodent models, the remaining functioning kidney also goes onto compensate for loss of function of the obstructed kidney. Thus, biomarkers of renal failure, such as serum urea and creatinine or proteinuria are often not clearly elevated using this model. Efforts to better reflect human disease, for example partial or reversible obstruction have been explored [24], but are not in widespread use due to their surgical difficulty therefore limiting the utility of this type of model.

**Surgical nephron reduction:** Sub-total nephrectomy mimics the consequences of reducing functional renal mass. It is most often used in rats and encompasses two different methodologies. The first is a ligation model where one kidney is removed with consequent ligation of polar branches of the renal artery of the contralateral kidney. In the other method, the rat undergoes a nephrectomy and then roughly 50% of contralateral kidney is excised 1-2 weeks later. The latter model does not tend to manifest with hypertension whilst the former does [25]. In both models the rats develop progressive renal failure by week 2 with renal histology showing progressive glomerular and interstitial fibrosis with renal dysfunction and proteinuria which is similar to that found in human disease [26]. Conversely mice are typically resistant to induction of chronic renal injury by nephron reduction [27] although there is substantial inter-strain variability [28]. Furthermore the challenges of carrying out surgery in small animals should not be underestimated [29].

**Uninephrectomy DOCA/salt models:** The mineralocorticoid deoxycorticosterone acetate (DOCA) when administered with a high salt diet and unilateral nephrectomy induces renal injury, low renin levels and hypertension in both rat and mouse models. It consists of a 2-6-week period of high salt and mineralocorticoid exposure. The DOCA itself is administered by either subcutaneous pellet insertion or oral supplementation. Animals then develop hypertension followed by proteinuria, glomerular sclerosis, tubulointerstitial inflammation and fibrosis in keeping with progressive CKD [30] although the severity of the phenotype varies between different rodent strains. For example, 129/SV mice have markedly higher DOCA-induced blood pressure, glomerulosclerosis, interstitial fibrosis and albuminuria compared to C57BL/6 mice [31].

This model has been used to investigate the relationship between the renin–angiotensin–aldosterone system (RAAS) activation and CKD [32] as well as the therapeutic effect of angiotensin converting enzyme inhibitors (ACE-I) [33]. The cardiovascular phenotype probably reflects only one of the potential mechanisms (i.e. sodium retention ± oxidative stress) through which kidney injury impacts on the CV system. Therefore, although this approach has been used extensively to study hypertension and the final common pathway of CKD it may not provide a comprehensive model for interrogating the fundamental mechanisms of CKD-associated CVD.

**Nephrotoxic Models of CKD:** Nephrotoxic models of CKD are attractive as they typically cause less suffering and are technically less challenging than surgical models. First proposed as a viable CKD model in 1982 [34], **adenine**, when administered in high quantities, saturates the adenine phosphoribosyl transferase pathway, causing it to be oxidised into 2,8 dihydroxyadenine by xanthine oxidase. 2,8-dihydroxyadenine is renally excreted and precipitates within the tubule leading to tubulointerstitial inflammation and fibrosis [35]. The adenine model leads to severe CKD, with marked biochemical renal failure and associated vascular calcification [36]. By adjusting the concentration of adenine in rat chow Shobeiri et al. were able to produce stable CKD at 5, 8 and 11 weeks [36]. Sex plays a key role in this model with female rats requiring higher adenine

concentrations in their diet to achieve the same severity of kidney injury [37]. The model has also been validated in mice [38]. Hypophosphatemia, secondary hyperparathyroidism, renal osteodystrophy and vascular calcification are prominent in this model [39], a phenotype consistent with the consequences of the CKD-mineral bone disorder (CKD-MB) observed in patients.

A single dose of 250mg/kg of **folic acid** causes an acute kidney injury in mice. Animals go onto form crystals within the tubular lumen causing acute tubular damage followed by tubulointerstitial fibrosis over 2 weeks [40]. By alkalinising the urine, using sodium bicarbonate, folic acid crystals can be reduced but tubular damage still occurs suggesting a direct nephrotoxic effect also contributes in this model [41]. Acute damage occurs within 2 weeks with the chronic fibrosis becoming apparent between 4-6 weeks. Biochemical markers of renal impairment occur in parallel with renal fibrosis [42] making this a particularly useful model for studying the consequences of CKD of different severity. This model is increasingly used, for example Rattanasinganchan et al. used this approach to investigate urinary biomarkers of tubulointerstitial fibrosis [43]

**Aristolochia** when given to animals (rats at a dose of 10 mg/kg daily subcutaneous injections for 35 days, for mice 3mg/Kg intraperitoneally every 3 days for 6 weeks) induces proteinuria, elevated serum creatinine with associated tubular necrosis, atrophy and interstitial fibrosis by day 35 [44,45]. These findings closely resemble those found in Chinese-herb nephropathy in humans a disease in which aristolochia has been shown to be the causative agent [46]. As such this model of human tubulointerstitial nephritis and has been predominantly used to study the molecular basis of kidney fibrosis rather than to investigate CKD-associated complications. However, given that albuminuria is a feature of this model, some studies have suggested that aristolochia may also lead to glomerular damage with podocyte effacement noted on electron microscopy [47]. This model has predominantly been investigated in mice (NMRI, FVB, C76BL/6, C3H/He strains have all been studied) and there is some evidence that there may be a degree of renal recovery after 9- 15 weeks [45].

**Immunological models:** Of the immunological models, the anti-thy1 model in rats has been most studied. Thy-1 is an antigen found on glomerular mesangial cells. When rats are injected with a single dose of either anti-thymocyte serum (containing anti-Thy1 antibodies) or a mouse anti-Thy1 monoclonal antibody, animals develop a glomerulonephritis [48]. Histologically there is initially mesangiolysis with an inflammatory cell infiltrate followed by mesangial matrix expansion with the occasional extracellular crescent [49]. This process takes roughly 1 week with subsequent repair from 3 weeks onwards. The renal phenotype of proteinuria, haematuria and renal impairment reflects the abnormalities seen in human glomerulonephritis (GN). Although in humans there is hypertension (as opposed to normotension in animals) and immune complex deposits histologically, that are not seen in the animal model. This model has also been combined with uninephrectomy to produce a GN model more typical of a glomerulonephritis associated CKD [50]. Note this model is not viable in mice.

**Other models:** We have only outlined the most commonly used approaches to induce renal injury in rodents. Many others are described, particularly those aimed at modelling specific diseases, these include: Spontaneous models of glomerular and interstitial injury such as NZB/W lupus nephritis model [51], or the Sprague Dawley rat aging model [52], spontaneously hypertensive rats (SHR) [53]; genetically engineered models such as the mouse Alports model lacking collagen alpha 3(IV) [54]; acquired immunological models such as models of anti-glomerular basement disease (anti-GBM)[55]; acquired non-immunological models such as radiation nephropathy [56] or cyclosporine nephropathy [57]. These approaches have been extensively reviewed elsewhere [16].

### **Cardiovascular phenotyping**

**Blood pressure** is the simplest cardiovascular phenotype and is typically elevated in CKD. Non-invasive methods involve using a tail-cuff. This can be performed on awake restrained rodents. However, restraint induces agitation in mice and rats unless they have been suitably conditioned and



tail-cuff readings generally correlate poorly with invasive measures [58]. Terminal invasive methods require the use of anaesthetic agents that may interfere with BP readings [59]. Conversely telemetry devices allow BP measurement during normal activity [60] and represent the gold-standard approach, however, this requires additional animal procedures, the surgery is technically challenging and equipment expensive. Blood pressure measurements have typically been performed in all the models described above. Hypertension is typically observed in the nephron reduction, DOCA-salt, folic acid and the adenine models, however, there are marked differences between species and strains [61].

**Pulse wave velocity (PWV)** using ultrasound non-invasively or invasively directly relates to the burden of atherosclerotic disease in both animals [62] and humans [63]. However it is also a key measure of arterial stiffness in the absence of atherosclerosis and therefore an important characteristic of CKD associated CVD. PWV is quantified in animals by measuring two pressures a known distant apart with high fidelity transducers and then determining the delay between waveforms. The adenine model exhibits increased PWV, this occurs early within 5 days of model induction [64] and continues as the animals vasculature becomes more calcified [65].

Abnormal endothelial function has long been linked to the pathophysiology of CKD associated CVD with a number of implicated mechanisms including alterations in nitric oxide (NO), asymmetric dimethylarginine (ADMA) and advanced glycation end products (AGEs) [66]. Hypertension is known to cause endothelial dysfunction and in nephrectomy rat models [67] and DOCA-salt models aberrant levels of endothelial mediators are found [68]. However, the folic acid [69], adenine [70,71] and UO [72] models have all been used to investigate mechanisms and potential therapeutics of endothelial dysfunction in CKD. Most simply these methods are *ex vivo* **transcriptomic** assays however, there have been some attempts to model *in vivo* **endothelial function** using high-resolution ultrasound to measure flow-mediated vasodilation in both rats and mice in response to different endothelial activators or inhibitors [73,74].

**Arterial histology** in CKD associated CVD typically demonstrates medial thickening or fibrosis along with calcification in the smooth muscle layer and this phenotype has been demonstrated in animal models. Initially the adenine model involved feeding the animals a high phosphate diet that drastically reduced the animals' weight and confounded results, however, an updated model involving a high initial adenine dose combined with a normal diet has resolved this issue [75]. However, in non-adenine models combining a high phosphate diet with the existing model can heighten the calcific phenotype [36]. Histopathology can also demonstrate accelerated plaque formation when examining models of atherosclerotic disease in the context of chronic renal injury.

Functional cardiac imaging is also possible in small animals. Patients with CKD-associated CVD typically demonstrate LVH, ventricular dysfunction and cardiac fibrosis. Teams such as Zhang et al [26] have utilised **echocardiography** to analyse systolic and diastolic functioning of the hearts in animal models of renal injury; revealing impairment in both. Cardiac **MRI** has also been used to illustrate cardiac hypertrophy and fibrosis in rodent models of CKD [76].

Others have demonstrated **ex vivo functional** changes for example, decreased contractility in a subtotal nephrectomy model in rats [77]. **Cardiac histology** (and weight) can also be used to demonstrate increased heart size and fibrosis. For example, the DOCA-salt model (rats and mice) also exhibit a cardiac phenotype in keeping with hypertension with increased heart size, cardiac fibrosis [68].

To what degree the above animal models of chronic kidney injury recapitulate the CV phenotype, other than hypertension and LVH, observed in patients has been inadequately explored in all but a few cases. However some of the above models do demonstrate vascular features suggestive of some aspects of arterial disease seen in CKD and are worth additional comment.

For example, the adenine model, alongside hypertension, mimics the CKD-MBD with evidence of arterial calcification and hyperphosphatemia and secondary hyperparathyroidism. Evidence from

this model suggests vascular smooth muscle cells transform into an osteochondrogenic-like phenotype [78–80].

Reduced NO bioavailability has long been suggested to underlie vascular pathology in CKD. Rats that have undergone subtotal nephrectomy, develop a worsening systolic function and cardiac fibrosis (both features of CVD-associated CKD) when treated with a low dose of a NO synthase inhibitor, providing support to this theory [81]. Similarly a nephrectomy rat model has been used to highlight the role of the uraemic toxin indoxyl sulfate in cardiac fibrosis, as well as test out monoclonal antibodies against cardiotonic steroids implicated in uraemic cardiomyopathy [82,83].

Therefore the evidence for mechanisms underlying the kidney-vascular link remain sparse and certainly there is no comprehensive understanding of the pathological pathways leading to the development of CKD associated CVD. This will not only require induction of a range of different models of CKD but the systematic characterisation of CV phenotype using whole organism physiological measures, imaging and interrogation of isolated tissues, across multiple time points.

#### **General considerations designing experiments based on animal models of CKD associated CVD**

A number of further considerations arise when planning experiments based on animal models of CKD-associated CVD, both general and specific to the research question. The generalizability of the model given the age, sex, species, and strain of the animals included in the experiment is fundamental to the conclusions being drawn. For example although experiments are typically performed in mice at age 8-12 weeks old, older animals [84] are likely to better reflect the pathophysiology observed in human CKD, a disease of middle and old age [85]. Similarly the sex of animals has been shown to influence the phenotype of models across a wide range of organ systems [86].

Species and strain is also important. Whilst mouse models are becoming more popular than rats due to the ability for genetic manipulation [87] and lower cost, many of the CKD models rely on surgical procedures and CV measurements which are technically less challenging in larger animals such as rats. Furthermore several strains of mice seem uniquely resistant to developing disease phenotype in several of the models described above (see Table 1). In addition rats used in experiments are typically outbred thus standardised nomenclature describing each strain e.g. “Wistar”, may be misleading as there can be a great deal of genetic heterogeneity within these broad strain descriptions [88].

Focusing on models of CKD associated CVD it is important to ensure that key CV findings are not related to the method of inducing kidney injury in the animal. Hence any proposed pathway or intervention should be examined in at least two differing models of CKD, e.g. one surgical and nephrotoxic model, and ideally across both rats and mice. Similarly the heterogeneity of human CKD must be recognised, again implying any important findings are replicated across surgical, toxic and immunologically induced models of renal injury. Finally the complexity of some of the procedures, particularly those related to CV phenotyping, means that only teams with extensive experience of performing this type of work can achieve high levels of reproducibility and minimise adverse events for experimental animals. To ensure compliance with the “three Rs” investigators without appropriate skills should ensure they collaborate with those that do.

## **Conclusion**

Although advances in technology may mean this approach becomes redundant, animal models will likely continue to be a useful method to describe pathophysiology and test potential therapies in CKD related CVD for decades to come. Thoughtful design of experiments, replication using different models and in-depth phenotyping using a wide range of techniques mean that we can maximise the benefits from these studies and over the longer term produce real benefit for patients.

**Table 1 CKD phenotyping in animal models**

Model	Method of Injury	Species	Strain	Sex differences	Time course	Renal Histological findings in animal	Translation to human disease	Other considerations	References
5/6 nephrectomy	Renal mass reduction	Rat/Mouse	Multiple	Males more susceptible	0-12 weeks	Glomerulosclerosis, mesangial expansion, tubulointerstitial fibrosis	Model for hyperfiltration injury e.g. renal cancer nephrectomy patients	Mice (esp C57BK/6) highly resistant Surgically complex Removed kidney is good control	[25–27]
Adenine	Tubular injury	Rat/Mouse	Multiple	Males more susceptible	6 weeks	Tubulointerstitial fibrosis, vascular calcification	Mimics CKD-MBD	Simple Rapidly progressive	[34,35,37–39]
Aristolochia	Tubular injury	Rat/Mouse	Multiple	None known	1-6 weeks post-dose	Tubulointerstitial fibrosis, potentially some podocyte damage	Aristolochia nephropathy	Simple Toxic nephropathy rare cause of CKD in humans	[44,45,47]
Folic Acid	Tubular injury	Rat/Mouse	Multiple	None known	1-6 weeks post-dose	Tubulointerstitial fibrosis, potentially some podocyte damage	Cyclosporine induced interstitial nephritis	Toxic nephropathy rare cause of CKD in humans	[40–42]
DOCA-salt	Renal mass reduction , vasoconstriction, hypertension	Rat/Mouse	Multiple rat, C57 mice	More severe in males	4 weeks	Glomerulosclerosis	Hypertensive CKD	Proteinuria, hypertension, low renin	[30–33]
Anti-Thy1	Immune-mediated injury	Rat/Mouse	Multiple	None Known	Immediate to 3 weeks	Mesangial necrolysis, mesangial	General model of glomerulonephritis with a	Needs nephrectomy to produce	[48–50]

						expansion, occasional crescents	predominant mesangial component	longer term phenotype	
UUO	Ligation of single ureter	Rat/Mouse	Multiple	None known	0-2 weeks	Hydronephrosis, tubulointerstitial fibrosis	Ureteric obstruction not common cause of CKD	No change in functional readouts Allows for internal control	[21,22,24]

**Table 2 CV phenotyping in animal models**

<b><u>Clinical CV phenotype</u></b>	<b><u>Measure in animals</u></b>	<b><u>Advantages</u></b>	<b><u>Disadvantages</u></b>	<b><u>References</u></b>
Blood pressure	NIBP	Simple	Not Reproducible Complicated by anaesthetic	[58–61]
	Terminal IBP	Simple	Sacrifices animal	
	Telemetry	Reliable and continuous	Surgery required	
Arterial stiffening and calcification	PWV	Can be done non-invasively	Cannot differentiate atherosclerotic disease from medial fibrosis and calcification	[36,62–64]
	Endothelial function	Allows for mechanistic approach to CKD-CVD	Whilst plays a part in human disease – not routinely measured in clinic practice	[67,68,74]

	Histology / transcriptomics	Allows for mechanism discovery		[68]
Atherosclerotic disease	Endothelial function	As above	As above	As above
	Histology	Better understanding of underlying pathophysiology		[36,75]
LVH	Heart weight	Simple	Coarse measurement	[68]
	In vivo imaging	Functional read-outs, more physiological. Read-outs can easily compare to human clinical practice	Technically challenging	[26,76]
Heart failure	In vivo imaging	As above	As above	As above
	Ex-vivo functional studies	Allows for easy variation of environment with functional readouts	Can only partially recreate true internal physiology	[77]
	Histology	Allows for investigation of final common-pathway	Sacrifice of animal	As above



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