# Evaluation of Cardiac Dysfunction in Patients with Chronic Kidney Disease

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## Intellectual Property and Publication Statements

The candidate confirms that the work submitted is his own and that appropriate credit has been given within the thesis where reference has been made to the work of others.

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# List of publications & presentations arising from this thesis

## Publications

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2. Chinnappa S, Hothi SS, Tan LB. Is uraemic cardiomyopathy a direct consequence of chronic kidney disease? Expert Rev Cardiovasc Ther. 2014 Feb;12(2):127-30.

3. Chinnappa S, Mooney A, Lewis NT, Goldspink D, El Nahas M, Tan LB. New evidence of cardiac dysfunction associated with renal impairment. Int J Cardiol. 2011 Nov 3;152(3):411-3.

## Oral presentations

- *National:* Chinnappa S et al. Evaluating Cardiac Dysfunction in Asymptomatic Chronic Kidney Disease Patients. UK Renal Week 2014, Glasgow, UK.

- *International:* Chinnappa S et al. Direct effects of a uraemic toxin on isolated rodent cardiomyocytes. ERA-EDTA Annual Congress 2014, Amsterdam, Netherlands.

**Poster presentations** (as the first author)

1. Chinnappa S et al. Does cardiac reserve improve with renal transplantation? ERA-EDTA 2017, Madrid, Spain.
2. Chinnappa S et al. Cardiac and Non Cardiac Determinants of Exercise Capacity in CKD. ERA-EDTA 2015, London, UK.
3. Chinnappa S et al. Evidence of cardiomyopathy in asymptomatic chronic kidney disease patients without primary cardiovascular diseases. ASN Kidney Week 2013, Atlanta, USA.
4. Chinnappa S et al. Does Uraemia per se Induce Cardiac Functional Impairment in Chronic Kidney Disease Patients? 4$^{th}$ Meeting of Uraemic Toxins and Cardiovascular Disease 2011, Groningen, Netherlands. **The poster was awarded first prize at the meeting.
5. Chinnappa S et al. Functional Reserve of Heart is Impaired in Chronic Kidney Disease Patients Without Primary Cardiac Disease. ERA- EDTA Congress 2011, Prague, Czech Republic.
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# Abstract

Heart failure (HF) is highly prevalent and associated with high mortality in chronic kidney disease (CKD). Although the cardiac structural alterations in CKD had been well studied, the pathophysiology of cardiac dysfunction in CKD, especially in the early asymptomatic stage, is not well understood. Identification of early cardiac dysfunction and an understanding of the pathophysiology of such dysfunction are vital in preventing the emergence and progression of HF in CKD.

The hypothesis underlying this thesis is that $CPO_{max}$ (peak cardiac power output) and cardiac functional reserve are impaired in asymptomatic CKD patients even in the absence of any known cardiac diseases or diabetes.

Asymptomatic CKD patients without primary cardiac diseases or diabetes mellitus were tested. $CPO_{max}$, a direct indicator of cardiac performance, was measured non-invasively using specialised cardiopulmonary exercise test. In addition, the reversibility of subclinical cardiac dysfunction after kidney transplantation was evaluated. Furthermore, to obtain mechanistic insights, the relationship between subclinical cardiac dysfunction and serum uraemic toxin concentrations, and the direct effect of a prototype uraemic toxin on the mechanical properties of isolated rodent cardiomyocytes were also evaluated.

Compared to healthy controls, the CKD patients showed a graded reduction in $CPO_{max}$ across different stages of CKD. The impairment was found to be reversible with kidney transplantation. The impairment correlated with total and free serum concentrations of indoxyl sulphate (IXS), a protein bound uraemic toxin. Further *in vitro* evaluation showed that IXS had direct physiological effects on cardiomyocytes and was shown to act through pathway involving protein kinase-A akin to the mechanism of action of sympathomimetics.

In conclusion, this *reverse translational* research has demonstrated for the first time that CKD per se causes impairment of peak cardiac power output and thereby cardiac functional reserve *in vivo*, and unravelled a novel mechanism of cardiotoxicity mediated by a protein-bound uraemic toxin *in vitro.*

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| | |
|---|---|
| **ACE-i** | angiotensin converting enzyme inhibitor |
| **AT** | anaerobic threshold |
| **BMI** | body mass index in $kg/m^2$ |
| **BNP** | B-type natriuretic peptide |
| **BSA** | body surface area in $m^2$ |
| **Ca** | Calcium in mmol/l |
| **CKD** | chronic kidney disease |
| **CMPF** | 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid in mg/dl; a protein-bound uraemic toxin. |
| **$CO_{max}$** | cardiac output in l/min at peak exercise |
| **$CPO_{max}$** | cardiac power output in watts at peak exercise |
| **CPX** | cardiopulmonary exercise test |
| **CVD** | cardiovascular disease |
| **eGFR** | estimated glomerular filtration rate ml/min. |
| **Hb** | haemoglobin in g/dl. |
| **HF** | heart failure |
| **$HR_{max}$** | heart rate in $min^{-1}$ at peak exercise |
| **IAA** | indole acetic acid (IAA) in mg/dl; a protein-bound uraemic toxin |
| **IXS** | indoxyl sulphate in mg/dl; a protein-bound uraemic toxin |
| **$MAP_{max}$** | mean arterial pressure in mmHg at peak exercise. |
| **PCG** | p-cresyl glucuronide (PCG) in mg/dl; a protein-bound uraemic toxin. |
| **PCS** | p-cresyl sulphate in mg/dl; a protein-bound uraemic toxin |
| **PKA** | protein kinase A |
| **$PO_4$** | inorganic phosphate in mmol/l. |
| **PTH** | parathyroid hormone in pmol/l. |
| **RER** | respiratory exchange ratio ($=VCO_2/VO_2$) |
| **RP8** | Rp8-Br-cAMPS |
| **SL** | sarcomere length |
| **SV** | stroke volume in mL |
| **UTox** | uraemic toxin |
| **$VO_{2max}$** | $O_2$ consumption at peak exercise |

# CHAPTER 1

## Introduction and Background

# INTRODUCTION & BACKGROUND

## 1.1 Overview

### 1.1.1 The role of kidneys

The first reference to kidneys in the western literature was by Aristotle (384-322 BC) in his works *Historia Animalium* and *De Partibus Animalium*. According to Aristotle, the kidneys' role was to separate surplus liquid from the blood to form 'residuum'. Through the works of Bellini and Malpighi in the 17$^{th}$ century, and Henle and Bowman in the 19$^{th}$ century a clear understanding of the structure of the kidney emerged. The discoveries over the last century helped improve our knowledge of the function of kidney and further research to clarify the finer functions of the kidney are still underway.

The kidneys' principal role is elimination of metabolic waste and the regulation of the volume and composition of body fluid. The functional unit of kidney is the *nephron* composed of *glomerulus,* formed by the invagination of a tuft of capillaries, and the *tubules.* There are approximately one million nephrons in each kidney and the kidneys receive nearly a quarter of the cardiac output. The hydrostatic pressure difference between the capillaries and the urinary space drives ultrafiltration at the rate of 120 – 130 ml/min which is the glomerular filtration rate. An estimate of this glomerular filtration rate (GFR) serves as a measure of kidneys' function. Kidney disease ensues when the kidneys' function drops and the degree of drop in GFR helps quantify the severity of the kidney disease.[1]

### 1.1.2 The interaction between kidney disease and heart disease

The first observations of the effect of kidney disease on the heart was by Richard Bright (1789- 1858) who documented that cardiac hypertrophy was a common anomaly resulting from chronic kidney disease.[2] The cardiac hypertrophy was thought to be a consequence of rise in blood pressure seen in kidney disease.

Alfred Stengel in 1914 proposed a definition for 'cardio-renal disease'

> *"When this combination of symptoms is of such character that the observer cannot readily assign to either the cardiovascular system or to the kidneys the preponderance of responsibility, the term "cardio-renal disease" is often employed".*[3]

In the same year (1914) Oskar Klotz wrote a review on cardio-renal interaction in the Canadian Medical Association journal titled 'The Triple Alliance: Heart, Kidney and Arterial Disease'.[4] In the article, the author described the lesions in the heart, kidney and arteries as 'sclerosis' and listed several possible mechanisms that led to the disease of the heart when kidney disease was the primary pathology. This included high blood pressure and direct chemical irritation on the heart by retained products of metabolism. The paper also highlighted the possibility of greater production of adrenalin in chronic kidney disease affecting the heart.

In 1940, Gouley first coined the term 'uraemic myocardiopathy'[5] and later Langendorf and Pirani showed that interstitial widening and fibrosis were common in hearts of patients dying from uraemia.[6]

The full extent of the problem of cardiovascular disease in chronic kidney disease and end stage renal disease was described in the 1990's through

epidemiological observations.[7-9] The studies showed that a large proportion of patients starting dialysis already suffer from cardiac abnormalities and dysfunction and consequently suffered a poor prognosis. In 2003, a statement from the American Heart Association (AHA) was published in Hypertension and Circulation underscoring the problem of increased cardiovascular risk in chronic kidney disease, and the lack of knowledge on pathophysiology.[10] This set the stage for the renewed enthusiasm in understanding the cardiac disease of chronic kidney disease over 150 years after its first description by Bright.

In this introductory chapter I shall present a review of current literature describing how kidneys' function/dysfunction is assessed and how kidney disease is classified into different stages of severity; what is the extent of heart disease in kidney disease; what is our current understanding of the nature of heart disease in kidney disease and finally highlight the gaps in knowledge.

## 1.2 Overview of Chronic Kidney Disease

### 1.2.1 Measuring kidney function/dysfunction

The filtering units of the kidneys, the glomeruli, filter approximately 180 l/day or ~125 ml/min which is called the glomerular filtration rate (GFR).[1] In health the GFR is maintained at a constant level due to autoregulatory mechanisms in the kidney. In disease, with a reduction in intrarenal blood flow, damage or loss of glomeruli or tubules, or obstruction to the free flow of ultrafiltrate along the tubules, the GFR will fall, and the ability to eliminate metabolic waste and to regulate the volume and composition of body fluid will decline. This will manifest as a rise in the blood levels of various solutes such as urea, creatinine etc and reduction in *measured* GFR.

The GFR can be obtained by measuring the excretion and plasma level of a substance that is freely filtered through the glomeruli and neither secreted nor reabsorbed by the tubules. The amount of such a substance in the urine per unit time must have been produced by filtering exactly the number of millilitres of plasma that contained this amount. This value is called the *clearance* for the substance. Inulin, a polymer of fructose, meets the criteria for a substance that is freely filtered and neither secreted nor reabsorbed and inulin clearance has been extensively used in experimental setting to measure GFR. In the clinical setting, the endogenous creatinine clearance serves as a convenient alternative. Creatinine is a product of degradation of creatine phosphate in muscle. It is generated at a constant rate in the body and excreted by the kidneys. Although some creatinine is secreted by the tubules and some absorbed by the tubules, the creatinine clearance values agree closely with that of inulin clearance. The GFR in a normal sized man is approximately 125 ml/min and its magnitude correlates well with the body surface area.[1]

As 24 h urine collection can be cumbersome, several equations are utilised nowadays to produce an estimated GFR (eGFR). The estimation equations incorporate clinical and demographic variables such as age, gender, race or lean body weight. The Modification of Diet in Renal Disease (MDRD) formula with 4 variables (serum creatinine, gender, race and age) is widely used in clinical practice and was utilised to estimate GFR in the present study as well.[11] Limitations in using eGFR include conditions where there is altered creatinine production (vegetarian diet, reduced muscle mass etc) and in some patient groups such as diabetics with high GFR, pregnant women, patients with morbid obesity etc. In these settings

creatinine clearance or clearance of an exogenous marker may be required to assess kidney function.[11]

### 1.2.2 Definition and classification of chronic kidney disease

The Renal National Service Framework has adopted the definition and classification of chronic kidney disease (CKD) proposed by US National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF-KDOQI).[12] This classification divides CKD into five stages (Table 1.1) defined by evidence of kidney damage and level of renal function as measured by glomerular filtration rate (GFR).

**Table: 1.1** Stages of chronic kidney disease

| **Stage** | **Description** | **GFR (ml/min/1.73m2)** |
|---|---|---|
| 1 | Kidney damage with normal or increased GFR | ≥90 |
| 2 | Kidney damage with mild reduction in GFR | 60–89 |
| 3 | Moderate reduction in GFR | 30–59 |
| 4 | Severe reduction in GFR | 15–29 |
| 5 | Kidney failure | <15 (or on dialysis) |

Stages 3–5 may be defined by GFR alone, whilst stages 1 and 2 also require the presence of persistent proteinuria, albuminuria, haematuria or structural abnormalities. Stage 3 was later subdivided into 3a (GFR 45-60) and 3b (GFR 30-45).[12] Stage 5 CKD may be described as established renal failure. Patients are said to be in end stage renal disease (ESRD) when CKD has progressed so far that renal replacement therapy (regular dialysis treatment or kidney transplantation) may be required to maintain life. In clinical practice, the GFR measurements are estimated measurements (eGFR) based on serum creatinine concentration. The Modification of Diet in Renal Disease (MDRD) formula is widely used.[11] Since the commencement

of the present study, eGFR based on CKD Epidemiology Collaboration (CKD-EPI) equation[13] and classification of CKD into grades[14] instead of stages have been slowly gaining prominence.

### 1.2.3 Epidemiology of CKD

Information on the prevalence of CKD in the UK is limited. However, data from National Health and Nutrition Examination Surveys (NHANES) in the USA gives an idea of the prevalence of CKD in general population. Data from the NHANES 2003 to 2006 database, with GFR estimated with the CKD-EPI equation, showed that the overall prevalence of CKD stages 1 through 5 is 14.2 percent.[15] Data from a large primary care study (NEORICA) in the UK suggests an age-adjusted prevalence of CKD 3-5 of 8.5%.

### 1.2.4 Complications of chronic kidney disease

The common complications of CKD include disorders of fluid and electrolytes, mineral and bone disorder, anaemia, hypertension, dyslipidaemia, endocrine abnormalities and cardiovascular disease. The relative risks of such complications increase with increasing severity of CKD.

### 1.2.5 Heart-kidney interaction

Although an association between cardiac and renal disease was observed in Richard Bright's seminal work,[16] research over the past 2 decades has uncovered several facets of this interaction and culminated in the classification of such interactions into 5 distinct types of cardio-renal syndromes (CRS) (Table 1.2).[17]

**Table 1.2** Classification of cardio-renal syndromes

| **Syndrome** | **Definition** |
|---|---|
| Acute cardio-renal (type 1) | Acute worsening of heart function leading to kidney injury and/or dysfunction |
| Chronic cardio-renal (type 2) | Chronic abnormalities in heart function leading to kidney injury or dysfunction |
| Acute reno-cardiac (type 3) | Acute worsening of kidney function leading to heart injury and/or dysfunction |
| Chronic reno-cardiac (type 4) | Chronic kidney disease (CKD) leading to heart injury, disease and/or dysfunction |
| Secondary CRS (type 5) | Systemic conditions leading to simultaneous injury and/or dysfunction of heart and kidney (e.g. sepsis amyloidosis, etc.) |

The present study focuses on type 4 CRS or chronic reno-cardiac syndrome where CKD, with its unique uraemic milieu, leads to the emergence of cardiovascular disease. The studies presented in this thesis specifically aims at improving our understanding of the *cardiac dysfunction* that occurs in CKD.

## 1.3 Epidemiology of cardiovascular disease in chronic kidney disease

### 1.3.1 CVD morbidity in CKD

CKD is a state of high cardiovascular morbidity and mortality. It was believed that the high prevalence of cardiovascular disease (CVD) in CKD is secondary to shared risk factors such as smoking, hypertension, diabetes mellitus, hyperlipidaemia, etc. However, CKD is increasingly being recognised as an independent risk factor for cardiovascular disease.[18-20] Cardiac disease of CKD originates early[18] and progresses relentlessly with increasing severity of CKD. The CV risk increases from 2 to 4-fold at stage 3 CKD to 10 to 50-fold at stage 5 compared to general population (Table 1.3).[21] The younger the patient, higher the relative risk.

**Table 1.3** CVD risk according to stages of CKD[21]

| Stages | CV risk (odds ratio) |
|---|---|
| 2 | 1.5 |
| 3 | 2 to 4 |
| 4 | 4 to 10 |
| 5 | 10 to 50 |
| ESRD | 20 to 1000 |

### 1.3.2 Different types of CVD in CKD

Data from United States Renal Data System (USRDS) shows the prevalence of different types of CVD in CKD and ESRD patients in US Medicare (≥ 65years) population (Table 1.4). Heart failure (as per coding for hospital admission with symptomatic heart failure) is the predominant cardiac abnormality of CKD with a prevalence of 44% in pre dialysis CKD population (Figure 1).[22] The commencement of dialysis does little to arrest the progression of heart failure and the prevalence rises up to 55% in end stage renal disease population (ESRD).[22]

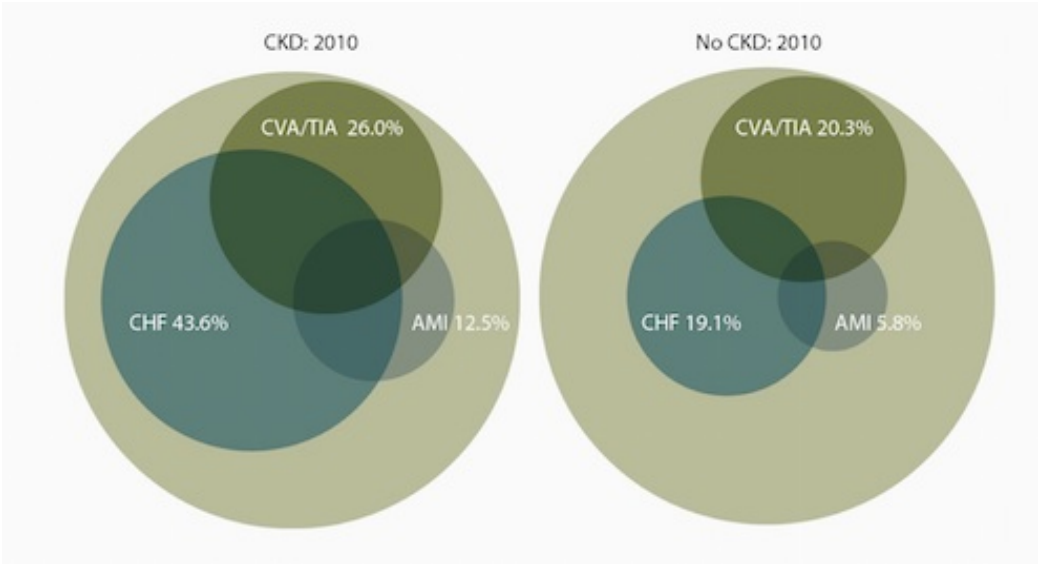


**Fig 1.1:** The figure shows the distribution of different CVD in CKD patients in the US Medicare (≥ 65years) population compared to general population. USRDS Annual Data Report 2012.[22] CHF: congestive heart failure, AMI: acute myocardial infarction, CVA: cerebrovascular accident, TIA: transient ischaemic attack.

**Table 1.4** Prevalence of CVD in different CKD stages

| CVD & CKD stages | Prevalence (%) |
|---|---|
| CHF | |
| Stages 1-2 | 26.8 |
| Stage 3 | 30.9 |
| Stages 4-5 | 40.9 |
| AMI | |
| Stages 1-2 | 9.7 |
| Stage 3 | 10.9 |
| Stages 4-5 | 12.9 |
| PVD | |
| Stages 1-2 | 24.1 |
| Stage 3 | 25.7 |
| Stages 4-5 | 29.6 |
| TIA/CVA | |
| Stages 1-2 | 18.1 |
| Stage 3 | 18.5 |
| Stages 4-5 | 20.2 |

Distribution of different CVD in CKD patients in the US Medicare (≥ 65years) population. USRDS Annual Data Report 2012.[22]
CHF: congestive heart failure, AMI: acute myocardial infarction, CVA: cerebrovascular accident, TIA: transient ischaemic attack.

### 1.3.3 CVD mortality in CKD

CKD is a state of very high mortality. The 5-year survival of men >64 years starting dialysis is worse than colon cancer or prostate cancer and the 5-year survival of women starting dialysis is worse than breast cancer or colon cancer.[23] More than half the deaths in ESRD population are attributable to CVD. In a large population-based study evaluating the life expectancy of over 1.5 million participants with varying levels of kidney function, it has been shown that the proportion of CVD deaths increase with increasing severity of CKD with 27.5% of CVD deaths in CKD 1&2 to 58% CVD deaths in stage 5 and ESRD (Table 1.5). [24]

**Table 1.5** CKD stages and CVD mortality[24]

| CKD Stages | CVD mortality (%) |
|---|---|
| 1 & 2 | 27.5 |
| 3a | 32.9 |
| 3b | 41.1 |
| 4 | 48.8 |
| Stage 5 and ESRD | 58.0 |

### 1.3.4 Adverse effects of CKD on pre-existing heart disease

In addition to the *de novo* heart disease in CKD, it is also important to understand the serious adverse effect CKD has on the outcomes of pre-existing heart disease. For example, in patients with acute myocardial infarction (MI), after adjustment for other risk factors, the risk of death or nonfatal cardiovascular complications increases significantly with declining GFR (hazard ratio 1.10 for each 10-unit decrease in GFR below 81.0 mL/min).[25]

The effect of renal dysfunction is even more pronounced on heart failure (HF) outcomes. Renal impairment is a common complication of patients with heart failure and a recent meta-analysis has shown that more than a quarter of patients with heart failure have moderate to severe renal impairment (eGFR<60mls/min) and the presence of renal impairment confers a serious adverse prognosis.[26] The same study showed that mortality worsened incrementally across the range of renal dysfunction with 7% increased risk for every 10ml/min decrease in GFR. The renal impairment in heart failure is not merely a marker of HF severity but is shown to be an independent predictor of survival in HF patients. Hence, eGFR forms an important component of predictive models evaluating the mortality risk of HF patients wherein the impact of eGFR on survival is shown to be as significant as age and NYHA class.[27]

**Summary of epidemiology:** CKD is emerging to be an important public health problem and CKD patients suffer from very high cardiovascular morbidity and mortality. The next section discusses the risk factors that lead to cardiovascular disease in CKD.

## 1.4 Pathogenesis of CVD in CKD

The risk factors for CVD and CKD can be divided into traditional risk factors and non-traditional risk factors (Table 1.6). Traditional risk factors are those that are associated with increased CV risk in general population and non-traditional risk factors are specific to CKD or more common in CKD. The traditional risk factors such as hypertension, dyslipidaemia, diabetes, older age, etc. tend to be clustered in the CKD population and therefore the influence of individual risk factors in the pathogenesis of CVD is difficult to delineate.

**Table 1.6** Traditional and non-traditional risk factors of CVD in CKD

| **Traditional risk factors** | **Non-traditional factors** |
|---|---|
| Older age | Albuminuria |
| Male gender | Uraemic toxins (especially protein-bound toxins) |
| Hypertension | Anaemia |
| Dyslipidaemia | Abnormal calcium/phosphate metabolism including |
| Diabetes mellitus | Extracellular fluid volume over-load and electrolyte imbalance |
| Smoking | Inflammation |
| Physical inactivity | Malnutrition |
| Obesity | Oxidative stress |
| Family history of cardiovascular disease | Sympathetic overactivity & Renin-Angiotensin-Aldosterone-System (RAAS) activation |
| | Fibroblast Growth Factor 23 (FGF23) |

### 1.4.1 Traditional risk factors

***Dyslipidaemia:*** In CKD, the prevalence of dyslipidaemia is influenced by the level of GFR and the severity of proteinuria. Low GFR is associated with high serum triglycerides (TG) and low HDL cholesterol levels. As proteinuria worsens, total serum cholesterol, LDL cholesterol and TG levels all increase and HDL cholesterol levels decrease.[28] Although there is high prevalence of dyslipidaemia in CKD, prospective data linking dyslipidaemia to CVD in CKD is limited. Most information is extrapolated from studies performed in the general population. Interestingly, however, a paradoxical relationship of low cholesterol with high mortality has been demonstrated in ESRD population.[29] Moreover, large randomised controlled trials (RCTs) of lipid lowering therapy in ESRD have either failed show any survival benefit[30,31] or at best shown only limited benefit.[32]

***Hypertension:*** The relationship between CKD and hypertension is bi-directional. Hypertension could be the cause of CKD in some instances and the effect of CKD in most situations. The presence and severity of hypertension in CKD can be influenced by several factors such as the etiology, duration and the severity of CKD. In a study of 1,795 patients with CKD, the prevalence of hypertension was shown to increase from 66% when the GFR was 83 ml/min to 95% when the GFR was12 ml/min.[33] In both diabetic and non-diabetic CKD patients high systolic blood pressure is associated with high CVD risk [34] and blood pressure control appears to confer survival benefit.[35]

***Diabetes mellitus:*** DM is the most common cause of CKD in the developed world and is strongly associated with CVD.[36] Although there is no randomized trial

evidence that demonstrates strict glycaemic control reduces CVD events, tight glycaemic control confers benefit by delaying the progression of kidney disease.[37,38]

***Obesity:*** In contrast to the general population, for which there is a U-shaped association between BMI and survival,[39] in haemodialysis patients incrementally higher BMI is associated with better survival [40] Similar survival paradox is also seen in CKD patients not yet on dialysis. [41]

***Smoking:*** As in the general population smoking has been shown to be associated with increased CVD risk in CKD.[42]

As risk scores based on traditional risk factors were insufficient in capturing the extent of CVD in CKD[43,44] and in view of the paradoxical association between some of the traditional risk factors and CV risk in CKD, there is growing interest in the non-traditional risk factors.

### 1.4.2 Non-traditional risk factors

The non-traditional risk factors of CVD include CKD-specific factors such as uraemic toxins, albuminuria, salt & water retention, CKD mineral bone disorder (CKD-MBD) and renal anaemia, and conditions common to most chronic illnesses such as malnutrition, inflammation and oxidative stress.[45] Some of these factors are better studied than others and the ongoing research continues to add novel factors to this list.

**Anaemia:** The prevalence of anaemia increases as kidney disease progresses and >50% of patients with a GFR less than 15ml/min have anaemia.[46] Anaemia of CKD is associated with left ventricular hypertrophy (LVH), left ventricular systolic dysfunction and cardiovascular morbidity and mortality.[47] [48] [49] Observational studies have found that for every 1g/dl decrease in haemoglobin (Hb) concentration, the combined risk of mortality, heart failure or developing LVH increases by 20–40% in

patients on chronic dialysis.[50]Although introduction of erythropoiesis stimulating agents (ESA) have improved the outcome of patients with advanced CKD, normalisation of Hb had not shown any benefits with regards to CV mortality.[51,52] Hence, most CKD guidelines recommend a Hb range of 10.5–12.5 g/dl for patients on ESA therapy.

**Albuminuria:** Albuminuria is not only a marker of kidney damage but also a strong predictor of CVD morbidity and mortality. This association persists even in patients without diabetes or hypertension.[53,54-56] The mechanism through which albuminuria exerts CV toxicity is not clearly understood. However, it is likely that albuminuria reflects endothelial damage. There is some evidence to show that therapies that reduce albuminuria (e.g. ACE inhibition) have beneficial effect on CVD morbidity and mortality in CKD.[57]

**CKD-Mineral Bone Disorder:** As CKD progresses, serum calcium levels are reduced and parathyroid hormone (PTH) and inorganic phosphate levels are elevated.[58] Several studies have demonstrated the association between raised phosphate, raised calcium and phosphate product and raised PTH and elevated CVD mortality.[59-61] It is proposed that the toxicity is mediated through vascular calcification.[62] Although no specific treatment strategy has benefit over the others,[63,64] most CKD guidelines recommend controlling phosphate and PTH levels. Fibroblast growth factor 23 (FGF23), an endocrine hormone that regulates phosphate metabolism, has recently been identified as a novel risk factor associated with CVD in CKD. FGF 23 has been shown to induce LVH in animal models. Furthermore, elevated levels of FGF 23 is associated with LVH in CKD patients.[65]

**Salt & water retention:** Chronic salt & water retention and overhydration are associated with poor CV outcomes in patients with CKD and ESRD.[66,67] The adverse

effects are believed to be mediated through vascular stiffening and left ventricular hypertrophy (LVH). The effects of hemodynamic alterations in CKD on cardiac structure are discussed in detail under the heading LVH.

**Oxidative stress and inflammation:** CKD is a state of high oxidative stress.[68] Oxidative stress is shown to cause vascular[69] as well as myocardial[70,71] damage and markers of oxidative stress are shown to be associated with left ventricular dilatation[72] and impaired left ventricular ejection fraction in heart failure patients.[73,74] In CKD, markers of oxidative stress are predictive of mortality[75] and clinical studies have demonstrated their association with atherosclerosis and vascular stiffening. [76,77]

CKD is a chronic inflammatory state, and inflammation becomes evident in the early stages of disease.[20] Markers of inflammation such as C-reactive protein (CRP) and interleukin 6 (IL-6) are elevated in patients with CKD and are associated with CVD.[78] However, benefits of anti-oxidant or anti-inflammatory therapy in reducing CV morbidity and mortality in CKD have not yet been shown.

**Renin-Angiotensin-Aldosterone-System (RAAS) activation**: The activation of the RAAS occurs inevitably as kidney disease progresses. This has been demonstrated both in animal experiments[79] and in humans.[80] Kidney ischaemia is suspected to be the central mechanism.[81] The deleterious effects of RAAS on the cardiovascular system have been well studied.[82,83] The survival benefits of RAAS inhibition have also been shown in CKD.[35] As a result, angiotensin converting enzyme inhibitors and angiotensin receptor blockers are the mainstay of treatment of CKD.

**Sympathetic over-activity:** CKD is associated with activation of the sympathetic nervous system.[84] In a study of over 200 patients undergoing haemodialysis, it has been shown that increased sympathetic activity is independently predictive of cardiovascular morbidity and mortality. The study employed plasma noradrenalin

(NA) levels as a measure of sympathetic activity and showed that, for every 1 nmol/L increase in plasma NA the risk of CV events increased by 8%.[85] The strong association between sympathetic activity and left ventricular hypertrophy in this patient group has also been demonstrated.[86]

Small RCTs have shown benefits of beta blockade in ESRD. Cice et al had shown, in a placebo-controlled RCT of 114 patients, that treatment with Carvedilol reduced CV mortality and hospital admission in ESRD patients.[87] In a recent open-label trial of 200 ESRD patients, Agarwal et al had shown that treatment with beta-blocker was better in reducing CV mortality and hospitalisation for heart failure compared to ACE-I.[88] However, beta-blockers still remain underused in this patient group.[89] Large randomised trials are needed to evaluate the safety and efficacy of β-blockers for the reduction of cardiovascular risk in patients with CKD.

**Uraemic toxins:** There is growing interest in the role of uraemic retention solutes,[90] accumulating in the blood as a result of kidney failure, as mediators of cardiotoxicity.[91] Uraemic retention solutes include: small water soluble compounds (MW< 500 Da) e.g. urea and creatinine that are removed by conventional dialysis; middle molecules (MW > 500 Da) e.g. $\beta_2$-microglobin that are better cleared by high flux dialysers, and protein bound uraemic toxins (PBUT) e.g. the indole, indoxyl sulphate (IXS) and the phenol, p-cresyl sulphate (p-CS). As PBUTs are mostly bound to albumin, they are not well cleared by dialysis. Dialysis is able to clear only the free fraction but not the albumin bound fraction and therefore their serum levels of PBUTs continue to rise in patients.[92]

It is becoming increasingly clear that PBUTs are associated with evolution of CVD in CKD, and IXS and p-CS are most highly implicated.[91] Vasculotoxicity of

IXS has been demonstrated by its ability to cause endothelial dysfunction, vascular calcification and induction of oxidative stress.[93-95] Vasculotoxicity of p-CS has also been shown.[96,97]

Clinical studies have shown that IXS and p-CS are associated with vascular calcification and IXS is also associated with vascular stiffening. Furthermore, these toxins are also shown to predict cardiovascular and all-cause mortality.[95,98] Therefore, it is of interest to study the direct cardiac effects of these toxins in addition to their vasculopathic effects.

In isolated cardiac tissue IXS caused fibrosis and hypertrophy in neonatal rat heart[99] where inhibition of AMPK signalling was implicated.[100] In adult rat hearts fibrosis and hypertrophy were linked to reactive $O_2$ species (ROS) production and NFκB signalling.[101] Reducing IXS levels by AST-120 (oral charcoal) reduced NFκB phosphorylation and fibrosis in 5/6 nephrectomy rats.[102] Fibrosis would be expected to hinder electrical conduction and predispose to arrhythmias. Although the above studies demonstrated the role of PBUTs in inducing cardiac ultrastructural changes, studies on the effects of these toxins on the mechanical and electrical properties of cardiomyocytes are still lacking.

**Summary**

In summary, the uraemic milieu comprises a unique set of factors with the potential to cause both vasculopathy and cardiomyopathy. In the next section I describe the structural and functional changes that the heart undergoes in the uraemic environment.

## 1.5 Pathology and Pathophysiology

### 1.5.1 Uraemic Vasculopathy

Although the focus of the thesis is Type 4 CRS i.e. the cardiac disease of CKD, it is essential to understand the vasculopathic effects of CKD as CKD patients are at a higher risk of coronary artery disease (CAD). Indeed, CKD is considered as a coronary artery disease risk equivalent i.e. CKD patients without known coronary heart disease (CHD) have a risk of subsequent cardiovascular events that is equivalent to that of patients with established coronary disease.[103] The first evidence of the unique characteristics of coronary artery disease in CKD was shown by Schwarz et.al. in 2000. [104] In this study, post mortem samples of coronary arteries from patients with end stage renal disease were analysed and compared with those of non-renal patients with CAD. It was found that coronary plaques in ESRD patients were characterised by increased media thickness and marked calcification. In addition, Nakano and colleagues demonstrated similar calcification of CAD in pre dialysis CKD patients. This study also showed that the frequency of calcified CAD lesions increased with falling renal function.[105]

Arterial calcification occurs in the tunica intima or tunica media of the vessel wall. Intimal calcifications are calcium deposits on atherosclerotic lesions and medial calcification occurs as a result of osteoblast transformation of medial smooth muscle cells.[106] The altered calcium, phosphate and PTH homeostasis that occurs in CKD appears to play a major role in the evolution of uraemic vasculopathy. Moreover, low levels of inhibitors of calcification (e.g. fetuin-A) also contribute to arterial calcification.[106] Clinically, detection of arterial calcification *in vivo* is undertaken by plain x-rays, CT scans and advanced tools such as electron beam CT and multi slice CT.[106] The presence of arterial calcification is a strong predictor of CV and all-cause

mortality in patients with CKD. The association has been found to be independent of traditional atherogenic factors.[107]

In addition to arterial calcification, arterial stiffening is also found to be highly prevalent in CKD.[62] Arterial stiffness is evaluated by measuring pulse wave velocity (PWV).[108] Traditional risk factors such as age and diabetes, and CKD related factors such as albuminuria and vascular calcification have been found to be associated with arterial stiffening.[109, 108] The clinical relevance of arterial stiffness in CKD was shown in a study of 241 ESRD patients with a median follow up of 72 months. The study showed that for each 1m/sec increase in PWV the relative risk of all-cause mortality was 1.39. [110] Increase in arterial stiffness is suspected to increase cardiac workload and shear stress of downstream vasculature leading to poor CV outcomes.[107]

As vascular calcification and stiffness are highly prevalent in CKD and are predictive of mortality they were utilised as surrogate end points in studies aimed at prevention of CVD in CKD. For example, in an RCT of 360 ESRD patients, cinacalcet, a treatment for secondary hyperparathyroidism, was shown to reduce vascular calcification.[111] However, a recent large RCT of over 3800 CKD patients with moderate to severe secondary hyperparathyroidism, showed that treatment with cinacalcet conferred no benefits in terms of cardiovascular mortality.[112]

These series of negative trials in CKD, using conventional and novel therapies for vasculopathy that failed to offer survival benefit highlights the complexity of CVD in CKD, and emphasises the importance of improving our understanding of the cardiomyopathic aspect of cardio-renal interaction.

### 1.5.2 Uraemic Cardiomyopathy

A brief description of cardiac hypertrophy in general and the associated pathophysiology is presented here before discussing uraemia-specific myocardial abnormalities.

The weight of human heart increases from 20 g at birth to nearly 350 g at adulthood.[113] Further increase in heart weight can occur due to extrinsic stimuli such as increased work load, RAAS activation, cytokines, etc or intrinsic genetic abnormalities.[114] This is called as cardiac hypertrophy. Although the term 'hypertrophy' refers to increase in the volume of tissues due to increase in cell size, cardiac hypertrophy in most situations involves both increase in cardiomyocyte size and number, and increase in the number of connective tissue cells i.e. hyperplasia. The cardiac hypertrophy can be associated with improved cardiac function and energy use when it is referred as 'physiological' hypertrophy or impaired cardiac function and energy use when it is called as 'pathological hypertrophy'.[115] Another important characteristic of physiological hypertrophy is the reversibility.[116] Physical exercise and pregnancy are examples where physiological hypertrophy occurs whereas hypertension and aortic stenosis are situations where pathological hypertrophy occurs.[114] It has been shown that in pathological hypertrophy the ultrastructural composition of the myocardium is altered with disproportionate increase in fibrous tissue. A study on human autopsy samples of pressure overload induced left ventricular hypertrophy (LVH) showed that the myocyte volume increased by 65% whereas the connective tissue cell numbers increased by 141%. There was an overall decrease in volume percentage of myocytes by 6%.[113] Such changes have direct consequence on cardiac function by impairing the ability of myocardium to contract (inotropy) and its ability to relax (lusitropy).[117] Furthermore,

the mediators of pathological hypertrophy also cause cellular and molecular abnormalities such as altered expression and function of contractile proteins, altered energy metabolism, abnormalities in excitation-contraction coupling, etc. leading to progressive myocardial dysfunction and heart failure.[118]

There are several different imaging modalities available to assess cardiac structure and function. The most commonly employed tool is echocardiogram (echo). Echo can help evaluate systolic and diastolic function, and estimate left ventricular mass. The widespread availability, portability and negligible risk are the advantages of echocardiogram. The following are the other imaging modalities that are used in special circumstances:

- Cardiac magnetic resonance (CMR) is more accurate and reproducible compared to echo. In addition to assessing cardiac structure and function CMR can also provide information on perfusion and fibrosis of the myocardium.[119]
- Radionuclide ventriculography (RVG), that involves exposure to ionizing radiation, can be used when there is significant wall motion abnormality or distorted geometry.[120]
- Cardiac CT helps in the evaluation of coronary arteries and characterization of myocardium in addition to assessment of cardiac function.[121]
- Single photon emission computed tomography (SPECT) is employed to assess myocardial perfusion in addition to cardiac function.[122]

### 1.5.2.1 Pathology

**Ultrastructural changes**

A study of post mortem examination of heart from dialysis patients provided the first glimpse of the ultrastructural changes of the uraemic heart.[123] The study showed that uraemia is associated with hypertrophy of cardiomyocytes and there is evidence of interstitial fibrosis. For comparison, the study analysed hearts from hypertensive patients and non-hypertensive control patients. The most significant finding of the study was that there is reduction in capillary length density in uraemic heart i.e. the capillary growth does not keep pace with the growth of myocardium and interstitium. This architectural abnormality has the potential to render the cardiomyocytes relatively hypoxic at times of increased demand. The authors suggested that activation of renin-angiotensin system and sympathetic overactivity could be potential mechanisms.

A recent study using isolated neonatal rat cardiomyocytes and fibroblasts demonstrated that indoxyl sulphate, a protein bound uraemic toxin, exhibited pro-fibrotic, pro-hypertrophic and pro-inflammatory properties after incubation for 48 hours in the cell culture.[99] This offers an additional mechanism of adverse cardiac remodelling in CKD in addition to the effects of RAAS activation and sympathetic stimulation.

**Morphological changes**

The most commonly employed tool for studying cardiac structural abnormality in CKD patients is echocardiography (echo). There had been several such studies and the most common finding is left ventricular hypertrophy (LVH).

The first of such studies was published in 1995.[124] In a prospective study of over 400 patients starting dialysis, Foley et.al. showed that 73.9% of the patients had

LVH at the time of starting dialysis. Left ventricular dilatation (LV cavity volume >90ml/$m^2$) was seen in 35.5% of patients. Only 15% of the patients had an echocardiographically normal heart. Age, male gender, arterial pulse pressure, blood urea level and serum albumin were found to be independently associated with LVH. Moreover, LVH was found to be independently predictive of mortality in these patients. It was also shown that the median time of development of heart failure in patients with LVH was 38 months.[125] Later studies have demonstrated the association between anaemia and LVH in CKD.[125]

The most recent study evaluating cardiac structure and function of over 3000 CKD patients established that LVH is the predominant structural abnormality of the heart.[126] The study showed that the prevalence of LVH increased with increasing severity of CKD. The prevalence was 32%, 48%, 57%, and 75% for eGFR categories >60, 45–59, 30–44, and <30 ml/min respectively. The severity of LVH, as measured by LV mass, was 46.1, 50, 52.5 and 57.8g/$m^{2.7}$ respectively. The relationship persisted even after adjusting for hypertension.

In addition to neurohumoral and toxin related mechanisms involved in LVH and fibrosis in CKD, preload and afterload related factors also play a role. The afterload-related factors include systemic arterial resistance, elevated BP, and impaired aortic compliance. [127,128] The last factor could be related in part to the aortic calcification and aortic stiffness seen in CKD and ESRD. These afterload-related factors result in myocardial cell thickening and on echocardiogram a concentric LV remodelling. Preload-related factors include expansion of intravascular volume (salt and fluid retention), anaemia, and, in certain circumstances, large flow arterio-venous fistulas placed for vascular access for haemodialysis.[129-131] These factors result in myocardial cell lengthening and on echocardiogram an eccentric or

asymmetric LV remodelling. Both afterload- and preload-related factors may operate simultaneously and probably have additive or even synergistic effects. Therefore, it is not easy to separate the effects of preload and afterload factors in the pathogenesis of LVH.

Cardiac MRI has also been employed to characterise the structural abnormality of uraemic cardiomyopathy (UCM). In a study of 134 dialysis patients using Gadolinium enhanced cardiac MRI, Mark et.al. had shown that LV mass is correlated with diffuse myocardial fibrosis.[132] The diffuse pattern of myocardial fibrosis was found to be unique to UCM, different from the sub-endocardial fibrosis associated with ischaemic heart disease.

As advanced CKD and ESRD is in most cases irreversible, LV mass may not be normalised in these subjects. However, appropriate fluid and blood pressure control and anaemia treatment results in some degree of reversal of LVH.[133] It has also been shown that in experimental uraemia and in a small observational study, reduction in circulating indoxyl sulphate, a non-dialysable toxin, was associated with some reduction in LV fibrosis and LV mass respectively.[132,134]

In summary, the predominant structural abnormality of UCM is LVH. From the available evidence, it is clear that LVH of UCM is characterised not only by cardiomyocyte hypertrophy but also by diffuse fibrosis suggesting a pathological remodelling. As heart failure is the predominant cardiac abnormality of CKD and LVH in CKD is a strong predictor of future heart failure, it is of vital importance to understand the functional consequences of this potentially pathological cardiac remodelling.

### 1.5.2.2 Pathophysiology

**Cardiomyocyte pathophysiology (cellular level)**

The heart is a transducer that converts chemical energy from nutrients into mechanical energy in the form of contractions. Cardiomyocytes are the functional units of the heart. The individual components of this transduction process such as substrate utilisation, energy production and generation of contractions have been studied in the uraemic milieu providing some useful insights into the working of uraemic heart.

*Substrate utilisation:* The altered architecture of the uraemic heart with its interstitial fibrosis and myocyte-capillary mismatch renders the environment of cardiomyocytes oxygen-poor. In such a setting the metabolism shifts from fatty acid utilisation to anaerobic glucose utilisation.[135] The inverse relationship between myocardial glucose utilisation and eGFR has been shown recently. It has also been shown that alteration in fatty acid utilisation has strong association with mortality in ESRD patients. [136]

*Energy production:* In 1993 Raine et al had shown, with the help of NMR spectroscopy, the effects of uraemia on cardiac energy synthesis in a uraemic rat model.[137] The study showed that, in rats rendered uraemic by 5/6$^{th}$ nephrectomy, the myocardial phosphocreatinine content was markedly reduced and there was also reduction in phosphocreatinine/ATP ratio. Similar changes were also shown in ESRD patients using NMR spectroscopy implying impaired energy synthesis in uraemia.[138]

*Cardiomyocyte contractility:* Two studies have shown the effects of uraemia on cardiomyocyte contractile function in the uraemic rat model. Both demonstrated that uraemia impairs contractile function of cardiomyocytes. There is impairment in myocyte calcium handling and as a result impaired relaxation. It was also demonstrated that the changes are independent of blood pressure. The authors proposed that these changes might translate as diastolic dysfunction *in vivo*.[139,140]

**Cardiac pathophysiology (organ level)**

As well as its use in cardiac structure, the most commonly employed tool for assessment of cardiac function in CKD is echocardiography (echo). The studies evaluating cardiac function in CKD included both ESRD (receiving renal replacement therapy) and pre-dialysis CKD patients. The impact of uraemia on both systolic and diastolic function was evaluated. The studies provided useful information on the extent of cardiac dysfunction in CKD and its significance on patient survival in CKD. A brief summary of some of the studies is presented here.

*Systolic dysfunction:* The analysis of systolic function by echocardiogram is usually performed by methods evaluating ejection phase, in particular ejection fraction. The first such evaluations were carried out nearly 2 decades ago. Early studies focussed mainly on advanced CKD and ESRD. A prospective study 432 patients starting dialysis showed 16% of the patients had systolic dysfunction (ejection fraction <50%) at the time of commencement of dialysis. [125] In the study, the median survival of patients with systolic dysfunction on starting dialysis was 38 months, with an odds ratio for mortality, compared to those with normal echocardiogram, of 1.88 (independent of age, gender, diabetes, and ischemic heart disease). In the most recent prospective study of advanced CKD patients followed through to dialysis, the ejection fraction was found to decline during the transition from CKD not requiring

dialysis to the dialysis-dependent stage. Moreover, the prevalence of systolic dysfunction (EF<50%) increased form 29% in the pre-dialysis stage to 48% in the dialysis stage.[141]

*Diastolic dysfunction:* Early studies evaluating diastolic dysfunction employed transmitral flow assessments.[142] As transmitral flow is considered load-dependant,[143] in recent years tissue Doppler imaging (TDI) of the mitral annulus or myocardial walls was introduced in the evaluation of segmental and global diastolic function.[144] Small observational studies have demonstrated the high prevalence of diastolic dysfunction in CKD and its association with mortality.[145,146]

Although the above studies demonstrated the existence of systolic and diastolic dysfunction in CKD, it is important to note that such studies included patients with concomitant cardiovascular diseases or risk factors of cardiovascular diseases such as diabetes. Moreover, the studies lacked sufficient power to control for such confounders. Therefore, it was difficult to delineate the effects of CKD per se on cardiac function. A recently published large population based study of over 3000 CKD patients tried to address such issues.[126] The study evaluated cardiac structure and function using echo in asymptomatic CKD patients of varying severity. The study demonstrated that LVH is the predominant structural abnormality of CKD and its prevalence and severity increased with increasing severity of CKD. With regards to cardiac dysfunction, systolic dysfunction was demonstrable only in 8% of the patients and there was no association between kidney function and systolic dysfunction. The study showed a large prevalence of diastolic dysfunction with only 29% of the cohort having normal diastolic function. The majority of the patients had mildly abnormal diastolic relaxation (62%), with a minority categorized as

moderately (8%) or severely abnormal (1%). However, there was no association demonstrable between kidney function and diastolic dysfunction. The authors concede that there was a high prevalence of mild dysfunction in all stages of CKD and hence they could not demonstrate a graded relationship between renal dysfunction and diastolic dysfunction.

Echocardiographic studies of UCM have helped establish LVH as the predominant structural characteristic of UCM with strong dose-response relationship with severity of uraemia. However, its corresponding functional correlate was not identifiable using resting echocardiographic assessments. Therefore, there remains an important unanswered question as to whether the cardiac remodelling in CKD is an *adaptive* physiological response that compensates for the altered haemodynamic conditions and chronic hypertension in CKD or a *maladaptive* pathology secondary to a cardiotoxic uraemic milieu. The important discriminatory feature between these two states would be the cardiac function. An adaptive cardiac remodelling would result in normal or supra-normal cardiac function whereas a maladaptive remodelling would result in impaired cardiac function.[115] As the parameters of resting cardiac function in CKD were poorly discriminatory in this regard, it would seem an alternative approach is needed.

## 1.6 The concept of cardiac power output

*"A heart is what a heart can do".*

*- Sir James MacKenzie (1853-1925)*

The heart is a pump that imparts hydraulic energy into the arterial system to maintain the circulation of blood. Without this energy the circulation would come to a standstill. In physics, energy per unit time is 'power' and hence the rate at which the heart imparts hydraulic energy onto blood circulation is the 'cardiac power output'

(CPO). If one can quantify the power output of the heart one gets a direct indicator of cardiac performance. CPO can be measured in watts as a product of the heart's flow output and arterial pressure. CPO measurements can be instantaneous measurements or steady state measurements. The former has been attempted in earlier studies using sophisticated, invasive techniques measuring cardiac power at a particular time point of cardiac cycle. [147,148] However, a more clinically applicable measurement is the average steady state measurement by utilising the steady component of the flow, the cardiac output (CO) and that of pressure, the mean arterial pressure (MAP).[149]

$$CPO = COxMAP$$

Such a fundamental measurement of cardiac performance allows us to delineate another important property of the heart, its reserve function. The heart does not function at a fixed power output. It can increase its performance at times of demand and each heart has a ceiling maximum power output ($CPO_{max}$) that is physiologically achievable. The difference between the resting performance and the peak performance of the heart is the cardiac functional reserve.

$$Cardiac\ Functional\ Reserve = CPO_{max} - CPO_{rest}$$

When a heart begins to fail, compensatory mechanisms will be activated in order to maintain the resting cardiac performance within as normal a range as possible. Its peak performance will be, however, compromised and diminished. It is this diminution of the cardiac reserve that represents the true pump dysfunction and needs to be measured to enable us to detect early cardiac dysfunction in CKD before the patient becomes symptomatic and also understand whether cardiac responses to uraemia are adaptive or maladaptive.[150]

### 1.6.1 Measuring CPO

The essential step in the measurement of CPO is measuring cardiac output. Cardiac output can be measured invasively or non-invasively. The invasive methods include the direct Fick method described by Adolph Fick in 1870[151] and the thermodilution technique described by Swan and Ganz in 1970.[152]

*Direct Fick method:* This method is based on the principle that we can calculate the blood flow through the lungs (and in turn the cardiac output), if we know the amount of oxygen uptake by the lung and the arterio-venous oxygen difference, using the following equation. This method involves right heart catheterisation for sampling mixed venous blood and peripheral arterial cannulation for sampling arterial blood.

$$Q_t = \frac{VO_2}{CaO_2 - CvO_2}$$

Where

Cardiac output ($Q_t$) is pulmonary blood flow in litres/minute

$VO_2$ is oxygen uptake in litres/minute

$CvO_2$ is the venous concentration of oxygen

$CaO_2$ is the arterial concentration of oxygen

*Thermodilution technique:* It is a form of indicator-dilution technique that involves right heart catheterisation, wherein the indicator is a small known volume of either dextrose or saline that is cooler than blood. It is injected as a bolus through the proximal port of the pulmonary artery catheter, where it mixes with blood in the right ventricle. The mixing lowers the temperature of intraventricular blood. As the blood flows past the distal thermistor port, the thermistor records the temperature change over time and can electronically display a temperature-time curve. The area under

this curve is inversely proportional to the flow rate in the pulmonary artery. This flow rate should be equal to cardiac output, assuming that there is no intracardiac shunt.

In both the above techniques a pharmacological stimulus such as dobutamine is employed to measure peak cardiac output.

**Non-invasive measurement of cardiac output:** Non-invasive techniques of measuring cardiac output include $CO_2$ rebreathing technique and foreign gas rebreathing technique. Physical exercise, using a treadmill or bicycle ergometer is used to stimulate the heart to measure peak cardiac output.

*$CO_2$ rebreathing technique:* This technique is discussed in detail in the methodology section. Briefly, an indirect Fick method is used in which blood concentrations of $CO_2$ in mixed venous and arterial blood are estimated indirectly from their partial pressure in the gas phase, with $CO_2$ as the indicator gas, to determine cardiac output. Two approaches have been used for the measurement of mixed venous $PCO_2$ during exercise: Rebreathing from a rubber bag that contains a low concentration of $CO_2$, and the equilibration method, which uses a bag that contains a high concentration of $CO_2$ in oxygen. The mixed venous $CO_2$ content is determined from the $CO_2$ tension, which can be estimated from the $CO_2$ tension curve as it gradually increases toward a limit during rebreathing of a known gas mixture (e.g., 5% $CO_2$ and 95% $O_2$). End-tidal $CO_2$ is taken as a measure of $CO_2$ partial pressure in alveolar gas and is representative of arterial blood $CO_2$. Carbon dioxide output is determined from the expired air sample.

*Inert gas rebreathing technique:* In this method, a soluble inspired gas such as acetylene or nitrous oxide is used for rebreathing. The mixed venous content of the

inert gas is taken as zero, and arterial partial pressure is assumed to be the same as in end-tidal air. The application of such methods that uses the inert gas rebreathing technique during exercise has been described in patients with heart failure.[153]

### 1.6.2 Clinical utility of CPO

Measured invasively or non-invasively the peak cardiac power output ($CPO_{max}$) appears to be a direct indicator of cardiac function and the best predictor of survival in patients with a failing heart. It has been shown that peak cardiac power output measured using dobutamine challenge was better at predicting survival in heart failure patients compared to resting haemodynamic indices.[149] Later in 2001 Tan et.al had shown that $CPO_{max}$ measured non-invasively, using $CO_2$ rebreathing technique, during cardiopulmonary exercise test (CPX), is the best predictor of survival in patients with heart failure compared to conventional indices.[154] The results have been reproduced in a later study using inert gas rebreathing technique.[153]

The major advantage of measuring peak performance of the heart is its ability to discriminate between health and disease states and stratification of severity of disease states. As shown in the following schematic diagram, in failing hearts, the resting performance of the heart is kept as normal as possible by compensatory mechanisms whereas the peak performance falls in proportion to the severity of underlying disease (Figure 2). Moreover, CPO serves as an overall index of cardiac performance by incorporating both volume generating capacity and the pressure generating capacity of the heart. In situations where resting cardiac assessments failed to delineate the pathophysiology, assessment of peak cardiac performance would serve as an alternative.

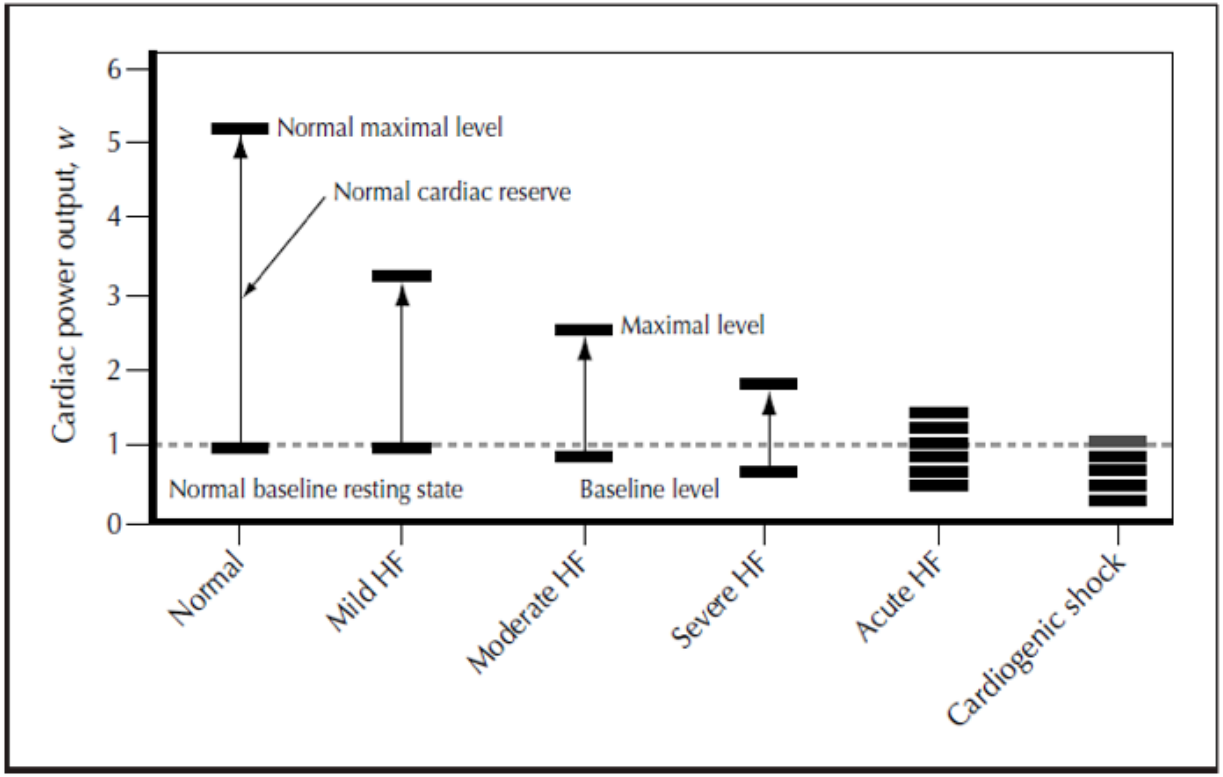


Fig 1.2: Schematic diagram depicting cardiac functional reserve of healthy heart in comparison with a spectrum of failing hearts. It demonstrates that the baseline performance of the heart is not as good as the peak performance in discriminating health and disease states.[150]

### 1.6.3 Assessment of peak cardiac performance in CKD

There have been few attempts at evaluating stress cardiac function in CKD. A study measuring myocardial contractile reserve in paediatric CKD patients, using exercise echocardiography, had shown that the contractile reserve is impaired even when resting parameters of cardiac function are normal.[155] In another study of paediatric CKD patients, peak oxygen consumption ($VO_{2max}$), a measure of physical functional reserve and a surrogate for peak cardiac performance, is shown to be impaired compared to healthy controls.[156] Since these paediatric populations are assumed to have minimal atherosclerosis, the findings suggest that CKD per se may have direct deleterious effects upon cardiac function. However, adult CKD patients without CV comorbidities have not yet been studied. Therefore, studies specifically designed to evaluate cardiac reserve in adult CKD patients without co-morbid atherosclerosis substrates are needed to obtain useful insights into uraemic cardiomyopathy. Studies measuring exercise capacity of adult patients with CKD and ESRD patients provide some useful insights.

### 1.6.4 CPX studies in CKD

CPX studies in CKD and ESRD patients have shown impaired physical functional reserve and a negative survival impact.[157,158] However, these studies did not exclude patients with IHD, diabetes mellitus or pre-existing HF and therefore it is difficult to ascertain whether the observations were primarily due to CKD or secondary to cardiovascular diseases. Moreover, there are several other factors that limit the utility of measures of exercise capacity such as $VO_{2max}$ or anaerobic threshold (AT) in assessing peak cardiac performance in CKD.

The question one needs to ask is ‘what do we measure when we measure exercise capacity in CKD patients?’ The claim that measures of exercise capacity could serve

as a surrogate of cardiac reserve function in CKD needs verification. The claim is based on the premise that $VO_2$ is a product of cardiac output (CO) and arterio-venous difference in oxygen concentration [$VO_2$= CO x $C(a\text{-}v)O_2$] and hence $VO_2$ could serve as a surrogate of cardiac output. There are several physiological considerations, commonly described in textbooks of exercise physiology,[151] which would render this simplified model less applicable in CKD.

- **Anaemia:** Oxygen is primarily transported as oxyhaemoglobin (and a small fraction dissolved in plasma) and it has been estimated that the $O_2$ carrying capacity of the blood falls from 22.5 ml/dL to 14.1 ml/dL as the haemoglobin concentration drops from 16 g/dL to 10 g/dL.[151] Hence, for a given cardiac output, the impaired $O_2$ delivery to exercising skeletal muscles, due to lower Hb, results in reduced peak $VO_2$ and anaerobic threshold (AT).
- **Chronic metabolic acidosis:** Exercising skeletal muscles generate acidic end products and the ventilation must keep in pace with the acid load to maintain normal pH. The presence of metabolic acidosis in CKD would add to the acid load and limit exercise capacity because of higher ventilatory requirements.
- **Peripheral vascular disease:** PVD is a common co-morbidity of CKD. The diseased vasculature with reduced internal diameter impairs blood flow to the exercising skeletal muscles. The relative ischaemia leads to early onset of lactic acid production and reduced AT irrespective of normal cardiac performance.[151]

- **Skeletal myopathy:** Muscle wasting is not an uncommon finding in ESRD. Reduced skeletal muscle mass limits the utilisation of delivered $O_2$. This coupled with the less studied phenomenon of skeletal myopathy secondary to uraemia would limit exercise capacity in spite of a normal cardiac output.[159]

It has indeed been shown that haemoglobin, serum albumin, co-morbid diabetes mellitus and cardiovascular disease are significant determinants of exercise capacity in dialysis patients.[160] Therefore this raises the possibility that exercise capacity in CKD is just a composite marker of co-morbidities rather than a true representation of cardiac performance. However, better interpretation of conventional CPX parameters in CKD can be achieved by employing techniques that simultaneously measure direct indicators of cardiac performance as well as exercise capacity.[161]

### 1.6.5 Measuring $CPO_{max}$ and cardiac functional reserve in CKD

The deleterious effects of the uraemic milieu on the heart, as discussed above, provides the physiological basis for the hypothesis that CKD *per se* would cause impairment of cardiac functional reserve. Furthermore, it is highly pertinent to note that CKD is associated with very high mortality in situations of high cardiac demand. The annual mortality secondary to sepsis is 30 to 45 fold higher in dialysis patients when compared to general population,[162] the one year post-MI mortality is more than 50% in dialysis patients[163] and CKD is also shown to have an adverse effect on survival during surgery.[164] Thus, on the one hand we have a set of mechanisms in CKD that could potentially cause impaired cardiac reserve and on the other hand we have evidence of the consequence of such impaired cardiac reserve in CKD. However, no evidence is hitherto available to measure and demonstrate diminished

cardiac functional reserve in CKD and this thesis discusses the first such evaluation of peak cardiac power and cardiac functional reserve in this patient group.

In this thesis, therefore, I defend the following hypotheses.

## 1.7 Hypotheses

The hypotheses to be tested in this thesis are as follows.

**Study 1**

- Hypothesis: Peak cardiac power and cardiac functional reserve are impaired in asymptomatic chronic kidney disease patients in the absence of any known cardiac diseases or diabetes mellitus.
- Aim: To measure peak cardiac power and cardiac functional reserve as a direct indicator of cardiac dysfunction in asymptomatic patients with diverse severity of CKD.

**Study 2**

- Hypothesis: Renal transplantation improves peak cardiac power in chronic kidney disease.
- Aim: To measure peak cardiac power in patients receiving kidney transplantation, before and after transplant surgery.

**Study 3**

- Hypothesis: Cardiac structure is altered in asymptomatic chronic kidney disease patients in the absences of any known cardiac disease or diabetes mellitus.
- Aim: To examine the relationship between echocardiographic measures of cardiac structure and the severity of renal dysfunction.

**Study 4**

- Hypothesis: Serum protein-bound uraemic toxins are inversely related to peak cardiac power in chronic kidney disease.

- Aim: To evaluate the association between serum levels of protein bound uraemic toxins and peak cardiac power in CKD.

**Study 5**

- Hypothesis: Indoxyl sulphate has direct inhibitory effect on mechanical properties of isolated rodent cardiomyocytes.

- Aim: To evaluate the physiological effects of indoxyl sulphate, a protein-bound uraemic toxin on the mechanical properties of isolated cardiomyocytes.

# CHAPTER 2

## Methodology

# METHODS

The overall aim of this study was to investigate the effects of chronic kidney disease on cardiac function. This chapter describes the common methodology relevant to the whole project and specific information is presented in the individual chapters.

## 2.1 Study design

***In vivo* study**

*Cross-sectional study* comparing cardiac function of patients with increasing severity of CKD and varying levels of the candidate biomarkers to a reference group of healthy volunteers as controls. Data from heart failure patients of New York Heart Association (NYHA) class II & III were used as positive controls.

- The CKD group (n=70): The group consisted of patients in CKD stage 2 to stage 5 (pre-dialysis).
- Healthy volunteers (n=101): Existing database of healthy volunteers was utilised for analysis.
- Heart failure patients (n=39): Historical data from the center were used for comparative analysis.

*Longitudinal study*: A subgroup of CKD subjects who underwent kidney transplantation during the study period underwent a repeat CPX study post-transplantation to assess the effects of kidney transplantation on cardiac function.

***In vitro study***

The acute physiological effects of the uraemic toxin indoxyl sulphate (IXS) was tested on freshly isolated rodent cardiomyocytes.

## 2.2 Setting

The *in vivo* study was conducted at the Yorkshire Heart Centre, Leeds Teaching Hospitals NHS Trust, Leeds, UK.

The *in vitro* experiments were conducted in the Faculty of Biological Sciences in University of Leeds, Leeds, UK.

## 2.3 Ethics approval

Ethical approval was obtained from South Yorkshire research ethics committee for all experiments (Ref: 11/H1310/8). All subjects received verbal and written explanation of the procedures involved and completed and signed consent forms.

## 2.4 Recruitment

Patients with CKD stages 2 to 5 were identified by a search of the renal database in Leeds Teaching Hospitals NHS Trust. Eligible subjects were approached in the outpatient clinics. Patient information sheets were provided and written consent obtained according to ICH GCP recommendations before participation. A total of 70 CKD patients were recruited for the study. The patients were recruited over a period of 3 years from a population of >1500 CKD patients under follow up in renal clinics. Sampling was determined only by the following inclusion and exclusion criteria and the willingness of potential participants to take part. Only patients able to understand spoken/written English sufficiently to provide signed informed consent to take part in clinical study were recruited. Exclusion criteria included patients unable or contra-indicated to perform exercise on a treadmill; patients whose exercise ability was limited by known significant musculoskeletal, cardiovascular, pulmonary, hepatic, neurological or other non-renal medical disorders. As per study design, to improve on previous protocols and exclude those with potential confounding factors

contributing to cardiac dysfunction, patients with any known cardiovascular diseases such as ischaemic heart disease, arrhythmia, valvular heart disease, peripheral vascular disease, cerebrovascular disease and renovasular disease, and diabetes mellitus were excluded.

## 2.5 Investigations

Baseline Characteristics: The age, ethnicity and cause and level of CKD/eGFR were recorded. Baseline cardiac assessment was performed in Renal and Cardiac outpatients and non-invasive investigation departments in Leeds Teaching Hospitals. This consisted of previous cardiac history, clinical examination, electrocardiogram, echocardiogram, biomarker assay and cardiopulmonary exercise testing (CPX). The cardiac history included any cardiac events or interventions, risk factors for cardiovascular disease and relevant medications. The clinical examination included formal cardiac and respiratory system examination, assessment of volume status and body mass index.

***Investigations:*** Information gained from standard investigations as part of good NHS clinical practice were recorded and not duplicated, such as routine full blood counts (FBC's), urea and electrolytes (U&E's) and estimated GFR measurements based on MDRD formula,[11] and other blood tests according to the individual patients' clinical needs (e.g. glucose, HbA1C, cholesterol, Ca++, phosphate, etc) and standard 12-lead ECG.

Apart from the above clinically indicated tests, the project-specific investigations for this study include the following: -

1. Cardiopulmonary exercise test (CPX)
2. Echocardiogram
3. Biomarker assays

### 2.5.1 Investigation 1: Cardiopulmonary exercise testing

Laboratory Conditions

All testing was performed in a dedicated cardiac power output laboratory in the Non-Invasive Unit of Cardiology, Leeds General Infirmary. The ambient temperature was maintained at 21ºC and the room was well-ventilated.

Subject Preparation

Prior to attending the laboratory for exercise testing, all subjects abstained from food and caffeine (3 hours) and alcohol (12 hours) to ensure that heart rate, blood pressure and flow measurements were undertaken without the influence of stimulants. Also, an exercise free period of 24 hours was required before each session to ensure a fully rested state, and hence maximal voluntary effort, on each treadmill test. During the visit to the laboratory, all volunteers underwent familiarisation with the equipment (treadmill, facial gas collection apparatus, gas cylinder contents for $CO_2$ rebreathing manoeuvres and CPX machine function). Any individual who had never used a treadmill was given instruction as to how best to walk without difficulty or placing undue strain on themselves. All procedures were described in detail, followed by discussion of any of the subject's queries or concerns. Any subject wanting to withdraw from the project was given the opportunity to do so before commencement of the tests or via the subject's voluntary termination during the test.

Any subject that did not complete the exercise test to maximal volitional effort was withdrawn from the data set. One of the participants was unable to walk on the treadmill and another participant developed ST-T changes during the CPX which was later investigated and found to be non-specific. Both the participants were withdrawn from the study.

CPX gas analysis system

Breath-by-breath gas analysis and measurements of cardiac output were performed using the automated Medgraphics Ultima system (Medgraphics Corporation, St. Paul, Minnesota, USA). This is a diagnostic exercise testing system that integrates both gas analyser and 12-lead ECG. Respiratory data were analysed and presented using Breeze Suite (version 5) and the ECG recording via Q-Stress Exercise ECG Testing System (Quinton Cardiology Systems, Inc.).

The Medgraphics gas analyser consists of a zirconia fuel cell that measures oxygen and an infra-red carbon dioxide analyser. The zirconia fuel cell is split into a sample and reference chamber. The cell is semi permeable to oxygen molecules and their movement generates a voltage that is measured by the cell, thereby allowing measurement of $O_2$ in the gas sample. Carbon dioxide absorbs infra-red light and the analyser is split into two chambers (reference and sample) through which beams of infra-red light are focused. The light absorption in the sample chamber is compared to that in the reference chamber, thus allowing quantification of $CO_2$ content.

The CPX gas analyser was calibrated prior to every test using both reference (21% $O_2$ and balanced $N_2$) and calibration (12% $O_2$, 5% $CO_2$ and balanced $N_2$) gases (Medgraphics Corporation, St. Paul, Minnesota, USA). System response time or "phase delay" was checked and then ambient $O_2$ and $CO_2$ measurements were checked using the inbuilt on-line calibration system.

The air-flow calibration was made via a pitot tube, attached at 90 degrees to a pneumotachometer (pre Vent, Medgraphics Corporation, St. Paul, Minnesota, USA) that measured the differential pressure of gas flow against 2 small tubes. The pressure was dependent on gas density and was therefore, sensitive to changes in gas composition. The pressure measurement was converted to air flow by first

establishing a zero-flow baseline, followed by 5 withdrawals and injections of air made at different speeds using a 3-litre syringe. This range of speeds simulated the varying respiratory rates observed throughout an exercise test. A correction factor, generated during the calibration, was applied to reduce the variability that exists between pneumotachometer readings.

Breath by breath gas sampling

A mouthpiece and saliva trap was connected to a T-shaped non-rebreathing valve and the mouthpiece positioned in the subject's mouth so as to create a perfect seal. A nose-clip was used to ensure 100% mouth breathing. The subject's air sample was passed through a drying cartridge before entering the gas module, to remove any moisture that may cause contamination of the infra-red window and hence, inaccurate gas quantification. Inspired and expired gas samples were analysed breath-by-breath for oxygen uptake ($VO_2$), carbon dioxide production ($VCO_2$), respiratory exchange ratio (RER), end tidal partial pressure of carbon dioxide ($ETpCO_2$), minute ventilation (VE), tidal volume (Vt) and respiratory rate (RR) using the Medgraphics CPX/D system (Medgraphics Corporation, St Paul, Minnesota, USA). Gas analysis output was presented as the mean 5 of 7 breaths to remove the confounding effects of large variations in ventilation.

Resting respiratory data

Respiratory data were collected continuously for 10 minutes prior to any cardiac output measurements. Data were further collected for at least 10 minutes between each measurement. These tests were performed in a standardized, quiet environment. If necessary, as indicated by high resting RER values or high VE values, the subjects were encouraged to slow the work of their breathing. If a subject requested a break

from the mouthpiece, a further 10 minutes of resting sampling was obtained prior to any measurements.

Resting and exercise ECG

Prior to the commencement of each test all subjects were fitted with a 12-lead electrocardiogram (ECG). A continuous 12 lead ECG was recorded throughout the exercise test to measure maximal heart rate (HRmax). Heart rate was determined directly from the R-R interval of the ECG.

Blood pressure measurements at rest and during exercise

Systolic and diastolic arterial blood pressure was measured at the left brachial artery via manual auscultation and sphygmomanometry. All measurements of blood pressure were taken in accordance with the British Hypertension Society guidelines.[165] The appropriate size cuff (width: 40-50%, length: 80% circumference of arm) was chosen for each subject and applied to the bare, upper arm to provide a firm but comfortable fit. The stethoscope was placed over the artery, just above the antecubital fossa; the cuff was inflated beyond the estimated systolic pressure, and then deflated at a rate of 2 mmHg per second. Systolic pressure was identified at the first Korotkoff sound (a repetitive, clear tapping sound for >2 consecutive beats) and diastolic at the fifth Korotkoff sound, after which all sounds disappeared.[166] Under resting conditions, blood pressure was taken in the seated position with the arm supported at heart level by the treadmill support bar and after at least 3 minutes rest to allow the subject's blood pressure to stabilise. During upright exercise, the subject's arm was supported, again at heart level, on the investigator's shoulder.

Carbon dioxide rebreathing techniques for measurement of cardiac output

The study subjects performed exercise upright on a treadmill. To enhance standardisation of testing, the participants were encouraged to use the hand rail and

to walk rather than run for as long as comfortable. They had a manual sphygmanometer cuff attached to their left arm for regular blood pressure measurement every 3 minutes. If a patient with CKD had an arterio-venous fistula on the left arm, the right arm was used instead. The subjects wore a 12 lead ECG which was monitored continually throughout rest, exercise and recovery. They breathed through the mouthpiece, enabling determination of gas exchange and flow with each breath. The bag was a non-latex anaesthetic bag, used to contain the carbon dioxide mixture. A valve is opened for the carbon dioxide measurements at rest and at peak exercise, so that they rebreathed from the bag rather than from room air for short periods during the rebreathing test.

*Cardiac output measurement*

Cardiac output was calculated by applying the indirect Fick equation:

$$Q_t = \frac{VCO_2}{CvCO_2 - CaCO_2}$$

Where

Cardiac output ($Q_t$) is pulmonary blood flow in litres/minute

$VCO_2$ is carbon dioxide production in litres/minute

$CvCO_2$ is the venous concentration of carbon dioxide

$CaCO_2$ is the arterial concentration of carbon dioxide

*CO2 production*: During cardiopulmonary exercise testing, $CO_2$ production was measured directly, along with $O_2$ consumption.

*Arterial $CO_2$ concentration:* It was assumed that there was equilibrium between the gas in the alveolar space and the pulmonary vein capillary bed. Therefore, the arterial $CO_2$ partial pressure can be extrapolated from the end-tidal pressure of $CO_2$ (ETpCO2) using the following equation[167]:

$$paCO_2 = 5.5 + 0.9(ETpCO_2) - 0.0021(VT)$$

Computerised dissociation tables then convert partial pressure to concentration.[168]

*Venous $CO_2$ concentration:* Calculation of venous $CO_2$ concentration involves re-breathing gas containing pre-determined concentrations of $CO_2$ until the gas in the bag and alveolar gas are in equilibrium (and therefore equal to venous blood).

This method of measuring cardiac output can be performed completely non-invasively with no risk as the only gas required is carbon dioxide. Several studies have been performed in the past using the technique.[154,169,170] Previous studies have confirmed the technique to be valid and reproducible,[171,172] and accuracy may be preserved with the automated calibration systems used in this study.

There are two methods for determining cardiac output; Collier [173] determined venous $CO_2$ from a plateau achieved when inspired and expired $CO_2$ partial pressures reach equilibrium after elevation of alveolar $CO_2$, achieved by re-breathing $CO_2$ from a bag at a higher concentration than the alveolar space. Defares [174] determined venous $CO_2$ concentrations by initially re-breathing a low concentration of $CO_2$. Here, equilibrium is not reached; venous $CO_2$ is calculated from the exponential rise in $ETpCO_2$ during re-breathing. Collier's method is more accurate for resting measurements whilst Defares' method is more accurate for peak exercise measurements. To improve the accuracy of the measurements, two measurements are taken for the resting CO and two measurements for the peak CO.

*The Collier method (equilibrium)*

The Collier method of rebreathing utilises a medical grade gas mixture comprising 10% $CO_2$, 35% $O_2$ and balanced $N_2$ to provide an initial partial pressure of $CO_2$ ($pCO_2$) greater than the subject's mixed venous $CO_2$ tension ($pvCO_2$). The high content of $O_2$ (35%) is sufficient to maintain normal arterial saturation throughout the rebreathing procedure. The gas mixture, at a volume 1.5-2.0 times the subject's resting tidal volume (VT), is re-breathed from a 5 litre anaesthetic bag via closed

circuit spirometry. The gases in the bag and alveoli mix, a fall in $pCO_2$ occurs resulting in equilibrium between the lung-bag system and $pvCO_2$ that is indicative of no further gas exchange. The equilibrium (seen as a plateau on the $CO_2$ graphical Breeze output) occurs between 8-12 seconds from the start of the re-breathing manoeuvre and must be maintained within 1mmHg for at least 2 respiratory cycles. At this point $pCO_2$ is assumed to equal $pvCO_2$.

Continuous end-tidal $CO_2$ ($ETpCO_2$) readings taken for the preceding 30 seconds before the start of the re-breathing manoeuvre provide a value for alveolar $pCO_2$ ($pACO_2$) from which arterial $CO_2$ tension ($paCO_2$) can be derived using tidal volume (VT) from the equation: paCO2 = 5.5 + 0.9 (ETpCO2) – 0.0021 (VT). For the calculation of cardiac output using the indirect Fick equation it is essential to convert the partial pressures of $CO_2$ ($paCO_2$ and $pvCO_2$) into the content of $CO_2$ ($CaCO_2$ and $CvCO_2$). This then allows determination of the difference in veno-arterial content ($CvCO_2$-$CaCO_2$) using the $CO_2$ dissociation curve for whole blood.

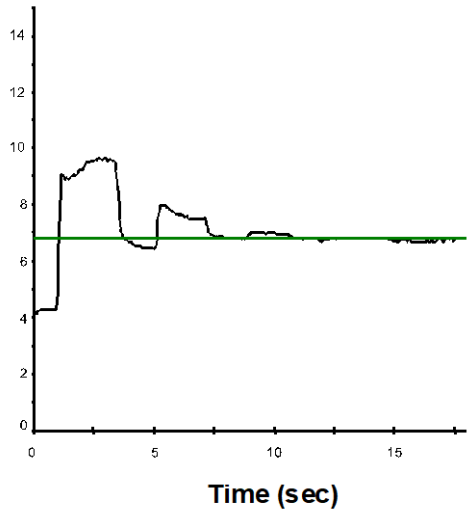


**Fig 2.1:** Equilibrium curve obtained at rest employing Collier's method of cardiac output determination.

*The Defares' method (exponential)*[174]

A gas mixture comprising 4% $CO_2$, 35% $O_2$ and balanced $N_2$ is utilised in this method for measurement of mixed venous $CO_2$ tension ($pvCO_2$). Once again, the high content of $O_2$ is sufficient to maintain normal arterial saturation throughout the rebreathing procedure and the gas mixture, this time at a volume approximately 1.0-1.5L greater than the subject's maximal tidal volume, is re- breathed from a 5 litre anaesthetic bag via closed circuit spirometry. The 4% concentration of $CO_2$ is lower than that of the subject's $pvCO_2$ and results in an exponential increase in $ETpCO_2$. A complete equilibrium between the lung-bag system and $pvCO_2$ is never attained, unlike that occurring during the Collier method, and $pvCO_2$ is mathematically calculated from the asymptotic rise in $ETpCO_2$. Calculation of $pvCO_2$ via this method during non-steady state maximal exercise has proven to be more accurate when compared to the Collier method,[175,176] and the lower $CO_2$ concentration is better tolerated by the subject.

Exercise testing protocol

The exercise tests were performed in three stages. The first stage was to measure resting CO, HR, $VO_2$ and BP. It was performed seated, after a resting period of at least 30 minutes. This test was performed when no significant exercise had taken place in the previous 24 hours. The second stage was an incremental test to determine the peak oxygen consumption ($VO_2$), heart rate (HR), blood pressure (BP) and anaerobic threshold (AT). This is performed by treadmill exercise according to the Bruce protocol.[177] The third stage is used to measure peak cardiac output (CO).

*Familiarisation and standardisation*

Patients were made familiar with the exercise equipment and introduced to the staff at the start of the study. The purpose of the studies and the procedures were

explained in full before written consent was obtained. The second stage of incremental exercise was always performed prior to the third stage.

This was therefore used as a familiarisation study. Where there is discrepancy between the results of the second and third stages, the third stage results were used to reduce the effect of familiarisation with the equipment and testing protocol. From the two tests performed at each exercise session, the higher $VO_2$ value from either test was used.

Exercise testing was conducted on the same Marquette 2000 treadmill (Marquette Electronics, Milwaukee, USA) throughout the studies, using standard Bruce protocols.[177] The same supervisors conducted the tests throughout. All tests were performed three hours postprandial.

*Calibration*

The gas analysis system was calibrated before each test. The pneumotachograph, which is used to measure gas volumes, was calibrated manually with a 3-litre syringe (Cardiokinetics, Salford, UK) by five injections and withdrawals, representing fluctuations in respiration, after a no–flow baseline is established. The $O_2$ and $CO_2$ analysers were also calibrated using bottled gases. The ambient room pressure was checked daily with a calibrated barometer.

*Stage one - Resting test*

Subjects rested for 10 minutes in the sitting position, to allow HR, BP and respiration stabilise. A mouthpiece was positioned in the subject's mouth with a nose clip to ensure mouth breathing. Further resting time was allowed once the mouthpiece was in position, to allow for any changes in respiration due to anxiety. To standardise each test, the measurements were taken when the minute ventilation (VE) was less than 10 L and the end-tidal $CO_2$ greater than 30 mmHg. Continuous respiratory, HR

and ECG (3 lead) monitoring was performed. Resting CO was then measured using the $CO_2$ re- breathing method of Collier as previously described. A 5 litre anaesthetic bag was connected to the pneumotachograph via a three-way tap. The bag was filled with 10% $CO_2$ 35% $O_2$ and balanced nitrogen, to a volume of twice the subject's resting tidal volume.

At end expiration, the three-way tap was altered so the subject breathed from the gas in the closed anaesthetic bag. The concentration of $CO_2$ in the bag exceeds the subject's concentration of $CO_2$ in their venous (pulmonary capillary) blood and the diffusion gradient between the venous blood and alveolar space are reversed. Equilibrium is achieved over four to five breaths. The computer is programmed to recognise equilibrium at the point where the difference between inspired and expired $CO_2$ is less than 0.1% in two successive breaths. Calculation of the partial pressure of $CO_2$ in the venous blood is automatically made and CO is calculated using the indirect Fick equation. At least three measurements of CO are made in order to calculate an average. Each test is performed several minutes apart, to allow for the washout of $CO_2$ from the circulation and to let the resting VE and $ETpCO_2$ return to stable baseline. Resting BP is measured initially, and then following each CO measurement. HR is measured continuously, but is recorded at the start of each CO measurement. Resting CPO involves measuring both BP and CO in a seated position.

*Stage two – Incremental test*

A mouthpiece was positioned in the subject's mouth with a nose clip to ensure mouth breathing. A minute of standing rest was given before starting the incremental test to allow the subjects to get used to the mouthpiece. $VO_2$ ($mls.min^{-1}$), $VCO_2$ ($mls.min^{-1}$), end tidal partial pressure of carbon dioxide ($ETpCO_2$, mm Hg), tidal volume (VT, L), and respiratory rate (RR, $min^{-1}$) were recorded breath-by- breath using the

Medgraphics CardiO2 analytic system (Medical Graphics Corporation, St Paul, Minnesota, USA). Respiratory exchange ratio (RER = $VCO_2/VO_2$), minute ventilation (VE = VT x RR, $l.min^{-1}$), and $VO_2$/kg ($VO_2$/weight, $mls.kg^{-1}.min^{-1}$) were calculated from the above variables.

Predicted peak $VO_2$ was calculated by the Wasserman technique for males: weight x [50.72 – (0.372 x age)].[178] Various ventilatory indices were calculated from the above data: ratio of minute ventilation to $O_2$ consumption at 1 litre of $O_2$ ($VE/VO_2$ 1l $O_2$) and at peak exercise (peak $VE/VO_2$), ratio of minute ventilation to $CO_2$ production at peak exercise (peak $VE/VCO_2$). The regression slopes relating minute ventilation to $O_2$ consumption ($VE/VO_2$ slope) and $CO_2$ production ($VE/VCO_2$ slope) were also calculated. The V-slope method[179] was used to calculate the anaerobic threshold (AT).

Subjects were verbally encouraged to exercise to exhaustion, and their limiting symptom (e.g. breathlessness or leg fatigue) was recorded. All patients performed symptom-limited exercise tests unless termination was indicated for safety reasons e.g. exercise induced hypotension or significant cardiac arrhythmia. Attainment of an age-predicted heart rate was not used as a criterion for stopping the test. The Borg score of perceived breathlessness and leg fatigue was checked every 3 minutes as a safety check because the subjects are not able to talk, and to gauge perceived exertion.[180]

A 12-lead electrocardiogram (ECG) was monitored throughout, and recorded at baseline, every 3 minutes and to record any abnormalities, e.g. ventricular ectopic beats. Blood pressure was measured manually using a sphygmomanometer (first Korotkoff sound used for systolic BP and fifth sound for diastolic BP) at baseline, two minutes into each stage, at peak exercise and during recovery. A maximal test

was considered if the respiratory exchange ratio (RER) is greater than 1 or if there is a plateau in $VO_2$ despite further increases in workload.

*Maximal exercise testing*

In terms of true exercise physiology, the definitive ascertainment of $VO_{2max}$ requires a levelling off of peak $VO_2$ despite increasing intensity of exercise.[181,182] It is widely recognised that this is achievable only in elite athletes, and unattainable even in healthy normal subjects, especially those who are sedentary and unaccustomed to endurance exercises. To set this as a necessary condition of determining true maximal exercise would be unrealistic when studying sedentary subjects. The peak $VO_2$ obtained during an incremental exercise test gives a reasonable approximation of $VO_{2max}$ for that exercise model. For the purposes of these investigations, we defined $VO_{2max}$ to be the highest $VO_2$ attained at volitional maximal symptom-limited exercise.

Taylor[182] comments that some normal subjects, as well as patients who may have become fatigued, discouraged or debilitated, may not be willing to push themselves to the point which produces the maximal oxygen uptake. This may be a limitation of the study. We elected to let the patients reach their self-determined volitional maximum. Verbal encouragement was provided, but it was deemed unwise to excessively push any of the subjects. The necessary conditions required to determine whether peak exercise has been reached were (i) that the individual subject dependably stated that they had reached a volitional exercise limit, whatever the cause (e.g. dyspnoea, fatigue, exhaustion), and (ii) a peak RER of greater than 1.05. The other variables monitored would be subsequently used during analyses of data to determine comparability of exercise end-points in the contrasting cohorts, as an aid to correct interpretation of experimental data in order to consider the

pathophysiological mechanisms responsible. Stage two also serves as a familiarisation step for stage 3 when the non-invasive cardiac output measurement is carried out.

*Resting period between incremental and peak exercise tests*

A rest period of at least 30 minutes (usually more) was required between the two maximal exercise tests. To prevent hyperthermia, subjects were encouraged to drink water during the rest periods.

*Stage three - Peak exercise cardiac output*

The third stage was to measure peak cardiac output. Defares' method was used to calculate CO at peak exercise, again using the indirect Fick equation. Subjects performed a constant maximum workload exercise test for at least five minutes, targeted for the same $VCO_2$ as that obtained during the incremental test as it was found that this achieved a peak $VO_2$ of at least that obtained at maximum level during the first stage. Subjects were encouraged verbally throughout and were told the $VCO_2$ goal that they were trying to achieve, which could be seen on the screen. At peak exercise, the anaesthetic bag was filled with 4% $CO_2$, 35% $O_2$ and balanced nitrogen, to more than twice the patient's tidal volume. The three-way tap was adjusted so the patient breathed from the bag for 10 seconds in order for the computer to construct a graph of end tidal points. The treadmill speed was briefly reduced to ensure subject comfort, obtain peak blood pressure and change the contents of the bag. The participant then exercised back to his peak again and the measurement was repeated, in order to take an average. The computer applies a best-fit exponential curve to the points according to the technique of Heigenhauser and Jones [183] rejecting the first point, and using 8 seconds of re- breathing to create an

exponential curve. End tidal points were manually adjusted to obtain the most accurate exponential curve.

**Calculations**

- Mean arterial pressure (MAP) has physiologic and clinical importance as it represents the tissue perfusion pressure. Mean arterial pressure is calculated using the Meaney formula.[184]

$$MAP = DBP + 0.412(SBP - DBP)$$

SBP and DBP are systolic and diastolic blood pressures measured in mmHg.

- Cardiac power output (expressed in watts) is calculated from the equation described by Cooke *et al* [161]

$$CPO = k(Q_t x MAP)$$

k is the conversion factor into watts, k = 2.22 x $10^{-3}$.

- Cardiac functional reserve was calculated as the difference between peak and resting cardiac power output

$$\Delta CPO = CPO_{max} - CPO_{rest.}$$

- Stroke work (SW) was calculated as the product of stroke volume and mean arterial pressure.

- Systemic vascular resistance (SVR) was also calculated.

$$SVR = \frac{MAP}{Q_t} x 80$$

The conversion factor 80 is used to convert Woods unit (mmHg.min.$l^{-1}$) into dyn.sec.$cm^{-5}$.

### 2.5.2 Investigation 2: Echocardiogram

A subgroup of patients (n=54) who underwent CPX testing took part in the echocardiography studies. Echocardiography was performed by a qualified senior echocardiographer. The subjects were scanned within 8 weeks after CPX testing. On the day of the test, a 3-lead ECG was attached to the subject and then they were rested in the left lateral position for 10 minutes in a quiet, warm room. All studies were performed using a GE Vingmed VIVID 7 (Horten – Norway) and images were post-processed on a dedicated Echopac PC.

A 2-dimensional study was performed in the standard parasternal long axis and short axis planes followed by an apical 4-chamber, apical 5-chamber and apical 2-chamber planes to evaluate cardiac structure and left ventricular contractility. In the parasternal long axis M-mode studies were performed in the left ventricle, at the end of the mitral tips. A frozen M-mode image was used to measure ventricular wall thickness and chamber size, in end diastole (EDD) at the peak of the R wave on the ECG and end systole (ESD) at the point of closest approximation of the posterior wall and the interventricular septum. Left atrial size was measured at the widest point during atrial diastole. Left ventricular ejection fraction (LVEF) was calculated by Simpson's biplane method[185] and left ventricular mass index (LVMI) was calculated by Devereaux method.[186]

### 2.5.3 Investigation 3: Biomarkers

Sample collection: Blood samples were obtained from study subjects prior to exercise testing. The sample was immediately centrifuged and the serum is stored as 0.5 ml aliquots in -80°C freezer.

***Uraemic retention solutes*:** Indoxylsulphate, p-cresylsulphate, p-cresylglucuronide, indole acetic acid, hippuric acid, uric acid, β2M, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF), asymmetric and symmetric dimethylarginine (ADMA and SDMA) were assayed in Ghent University Hospital, Ghent, Belgium.[90,187]

Quantification of the suggested uraemic retention solutes

HPLC Analysis: Assays are performed on samples collected at the time of CPX. For determination of the total fraction, serum samples are deproteinized by heat (95°C, 30 min). After cooling (10 min on ice), samples are filtered through a Centrifree-filter (Millipore, Billerica, MA). The ultrafiltrate is injected onto the column. To determine the free fraction, untreated serum samples are filtered through Centrifree prior to heating. Indoxylsulphate, indole-3-acetic acid, p-cresylsulphate and p-cresylglucuronide are determined by fluorescence analysis, while hippuric acid, CMPF and uric acid are analysed by UV detection [Waters Alliance 2695 device (Waters, Zellik, Belgium) connected to a Waters fluorescence and a UV detector].

### 2.5.4 *In vitro* analysis

The study was conducted in the faculty of biological sciences at the University of Leeds, Leeds, UK.

**Cardiomyocyte isolation**

Single ventricular cardiac myocytes were isolated from male Wistar rats (approx.250g). Hearts were removed following sacrifice by Directive 2010/63/EU approved methods. Hearts were quickly removed and placed in a solution (modified Tyrode's solution) containing (in mmol/l): 130 NaCl, 5.4 KCl, 0.4 $NaH_2$ $PO_4$, 1.4 $MgCl_2 \cdot 6H_2O$, 5 HEPES, 10 glucose, 10 taurine, 20 creatine and 0.7 $CaCl_2$ (pH adjusted to 7.3 with NaOH). Excess tissue was trimmed and the heart was cannulated on a Langendorff apparatus via the aorta (Fig 2.2); through which solution was already flowing at a constant rate of 8 ml/min to prevent air bubble formation in the coronary circulation. The heart was held in place with an artery clip then surgical suture thread was tightened around the aorta and glass cannula, ensuring sufficient perfusion pressure. All solutions were held in reservoirs where they were continually gassed with 100% $O_2$ and the temperature maintained at 37°C.

The heart was perfused until the superficial vessels had cleared of blood. A second Tyrode solution lacking $Ca^{2+}$ and containing $Ca^{2+}$ chelating agent EGTA (0.5mM) was then perfused for 4 min to stop contractions. This is thought to protect cells during the isolation procedure by inducing closure of gap junctions and weakening intercellular connections. A third Tyrode solution containing 0.05 mM $Ca^{2+}$, 1 mg/ml collagenase (Type II, Worthington Biochemical, USA) and 0.1 mg/ml protease (Type XIV, Sigma, UK) was perfused for 7 minutes; this digests the extracellular matrix and allows cardiomyocytes to separate. Enzyme solution could

be recirculated should the heart require more time to digest, otherwise it was collected and used in subsequent steps of the isolation procedure.

The heart was cut down from the cannula and the LV and RV were carefully dissected from the septum and coarsely minced in separate conical flasks in enzyme solution. The ventricle tissue was shaken in enzyme solution in a 37°C water bath to loosen cell attachments. Every 4 minutes the tissue was strained through nylon mesh and the remaining tissue was returned to the flask and the procedure repeated to give 4 or 5 separate fractions of cells. The collected enzyme solution was centrifuged at 50 x g for 40 s forming pellet of cells. Centrifuging at this speed helps separate dead cells, which stay in solution, from live cells which form a pellet. The supernatant was discarded and the pellet resuspended in 1 mM $Ca^{2+}$ Tyrode and stored in a centrifuge tube at room temperature and used within 10 h. If the heart did not perfuse properly and became hypoxic or did not digest, the cells were not used for the study.

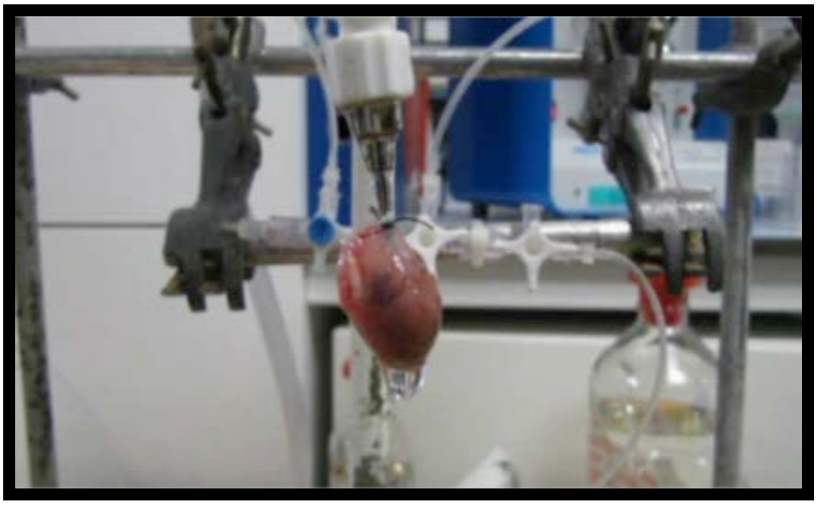

**Figure 2.2:** An example of the cardiomyocyte isolation set up where the heart is cannulated on a Langendorff apparatus

**Light microscope set up**

A drop of cells was placed in a superfusion chamber on the stage of an inverted microscope (Eclipse TE300, Nikon, Japan) fitted with a 40X objective. Cells were viewed through the eyepiece and a video image of the cells was acquired using a video camera (Myocam-S, Ionoptix, USA) mounted to the side of the microscope and displayed on a computer monitor facilitating simultaneous edge detection, sarcomere analysis and fluorescence experiments using IonWizard software (IonOptix, USA) (Fig 2.3). The cell bath was perfused with solutions in reservoirs suspended above the microscope which could be warmed by a heater prior to entering the cell bath.

**Figure 2.3:** An example of the light microscope set up to measure cardiomyocyte contractility. The reservoirs containing experimental solutions are not shown in this picture.

**Cell morphology and resting sarcomere length**

Cells were considered viable for experimentation if they appeared rod shaped with clear striations and were quiescent in the absence of stimulation. Average sarcomere length (SL) was measured by fast Fourier transform of an area of the cell, giving the dominant frequency of oscillations in contrast between light and dark bands which was automatically converted to length in microns in IonWizard.

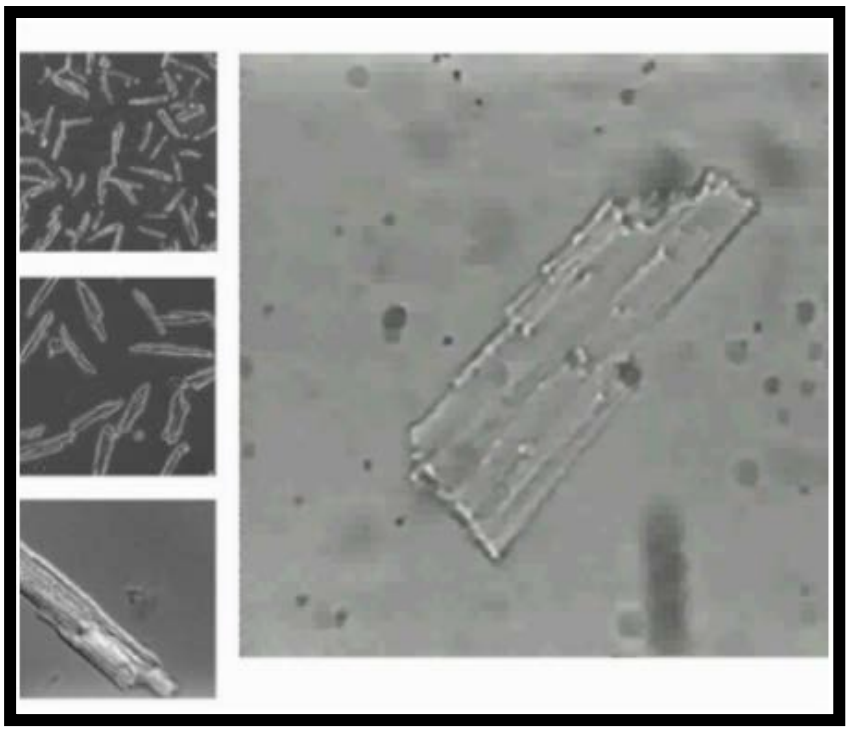

**Figure 2.4:** Example of viable a cell used for experimentation.

**Experimental Solutions**

The experimental (Tyrode) solution contained (in mmol/l): 137 NaCl, 5.4 KCl, 0.33 $NaH_2 PO_4$, 0.5 $MgCl_2 \cdot 6H_2O$, 5 HEPES, 5.6 glucose, and 1.8 $CaCl_2$ (pH adjusted to 7.4 with NaOH). Indoxyl sulphate (IS) (Sigma-Aldrich, Bornem, Belgium) was dissolved in saline to prepare the (300x) stock solution. The effect of indoxylsulphate (IXS) was assessed by switching the superfusion solution to Tyrode solution containing IXS at a final concentration of 4.49 mg/l, the maximum reported free concentration in uraemia. [188-190] IXS stock solution was tested for lipopolysaccharide contamination by a kinetic chromogenic Limulus amoebyte lysate test (Lonza, Vervier, Belgium).

**Measuring sarcomere shortening**

The cells were field stimulated via platinum bath electrodes using a 5-ms pulse at a stimulation frequency 1 Hz. Diastolic SL, systolic SL, fractional shortening (defined as a percentage change from diastolic SL) and the time constant of an exponential fit to the time course of the relaxation (Tau) were measured by averaging 10 – 15 twitches in each cell (Fig 2.5). The cells were initially superfused with the Tyrode's solution then switched to the solution containing IXS for at least five minutes exposure, then switched back to Tyrode's solution to assess the wash off effect.


**Figure 2.5:** A representative trace of sarcomere length change during stimulation at 1 Hz.

**Inhibition of Protein Kinase A**

To inhibit the action of Protein Kinase A (PKA), cells were incubated in storage solution with 100 µmol/l Rp8-Br-cAMPS (Rp8, Santa Cruz Biotech, USA), for at least 30 minutes.

**Measurement of calcium transients**

Intracellular calcium ($[Ca^{2+}]_i$) was simultaneously monitored with SL using the fluorescent $Ca^{2+}$ probe, Fura-2 AM (Molecular probes, USA). Isolated cells were loaded with Fura-2 AM (3.1 µmol/l for 10 min) at 20–23°C, re-suspended in Tyrode solution, and left for at least 30 min to allow de-esterification of the dye. Myocytes were placed in the experimental chamber and were alternately illuminated with excitation light at wavelengths 340 and 380 nm using a monochromator spectrophotometer system (Optoscope, Cairn Research, UK). Emitted light at 510 nm was collected by a photo- multiplier, and the ratio of emitted light in response to 340- and 380-nm illumination (340-to-380-nm ratio) used as an index of $[Ca^{2+}]_i$. The calcium transient amplitude was the difference between the diastolic and peak systolic 340-to-380-nm ratio. Myocytes were stimulated in Tyrode solution (Con) for 2 min before exposure to Tyrode plus IXS for 5 min then returned to control for 2 min.

**Fluorescence spectrophotometry**

To test for the possibility that IXS had a direct action upon the fluorescent indicator, Fura-2, the effect of IXS on the excitation spectra of Fura-2 was tested using the Optoscope. Fluorescence of Fura-2 free acid at 510nm, was measured in solutions containing (in mmol/l) 132 KCl, 1 $MgCl_2$, 1 EGTA and 10 HEPES together with 1 mmol/l EGTA and $CaCl_2$ at appropriate ratios to give the desired pCa.[191] The wavelength of the excitation light was increased from 300 to 400 nm in 5nm steps.

IXS in 140mmol/l NaCl was added to the experimental solution to give a final IXS concentration of 4.49mg/l and compared with that of the experimental solution with an equivalent volume of 140mmol/l NaCl.

## 2.6 Statistical analysis

Clinical study: $CPO_{max}$ was the primary outcome measure. The CKD 5 patients were slightly older compared to the controls. In order to correct for the age difference, $CPO_{max}$ is also presented as a percentage of predicted values for the age in addition to the absolute values. The percentage predicted values for the age was obtained from the historical control cohort of 101 sedentary healthy male volunteers. Comparison of $CPO_{max}$ of the controls and the study groups was performed using Independent sample t-test. The variations in $CPO_{max}$ and other study parameters across the study groups were analysed with analysis of variance ANOVA. The association between $CPO_{max}$ and various haemodynamic and biochemical variables were assessed by Pearson's correlation. As the exposure variables suffered strong co-linearity, their independent association with $CPO_{max}$ was assessed using partial least squares multiple regression analysis.[192,193] Results are presented as mean±SD (standard deviation). $P<0.05$ is considered statistically significant. SPSS 17.0 (IBM, USA) statistics software was used in the analysis.

Sample size: Based on the results of the pilot study of first 11 patients,[194] to demonstrate 10% difference in $CPO_{max}$ between study groups and healthy controls (with 90% power) a minimum of 11 patients per group was required.

*In vitro* studies: Data are expressed as means ± SEM (standard error mean). Statistical significance was tested using repeated measures ANOVA and independent sample t-tests where appropriate using SPSS statistics software version 17 (IBM, USA). Statistically significant difference was assumed when $P < 0.05$. Myocyte numbers for each experiment are given in the relevant figures/tables.

# CHAPTER 3

# Study 1

## 3.1 Introduction

This has been described in the full introduction chapter, but a brief description is given here. Heart failure (HF) is highly prevalent in chronic kidney disease (CKD) and confers a serious adverse prognosis.[195-197] Therefore, identification of early cardiac dysfunction and an understanding of the pathophysiology of such dysfunction are vital in preventing the emergence and progression of heart failure in CKD.

However, whether HF is a complication of the common co-morbidities of CKD such as ischaemic heart disease (IHD) and diabetes mellitus (DM), or whether CKD per se can cause cardiac impairment remains to be answered. Furthermore, it is not known whether asymptomatic cardiac dysfunction, the precursor of heart failure,[198] is present in CKD.

As described in the introduction, cardiac structural and ultrastructural changes in CKD have extensively been studied using techniques such as echocardiogram,[7] cardiac magnetic resonance imaging[132] and cardiac biopsy[123] offering valuable insights into uraemic cardiomyopathy by exposing abnormalities such as left ventricular hypertrophy (LVH), cardiac fibrosis and myocyte-capillary mismatch. However, attempts to detect cardiac dysfunction in asymptomatic CKD patients before they develop overt symptoms of heart failure have so far been unsuccessful. In a recent large echocardiographic study,[199] no relationship between *resting* measures of systolic and diastolic cardiac dysfunction and renal dysfunction in asymptomatic CKD patients was found. Alternatively, functional measures of *peak* performance of the heart have the potential to reveal subclinical cardiac dysfunction.

In Study 1 of this thesis, I tested the hypothesis that asymptomatic CKD patients have impaired peak cardiac power output and hence reduced cardiac functional reserve. Moreover, I tested CKD patients without primary cardiac disease or DM to study the effect of CKD-specific factors in isolation.

## 3.2 Methodology

Full methodology is discussed in Chapter 2. A concise methodology relevant to Study 1 is presented here.

Study subjects

Asymptomatic male patients (n=70) aged over 18 years, mean 48.4±12.6 years, with CKD stable for 3 months (stages 2 to 5) were recruited from the renal outpatient clinic in Leeds for cardiopulmonary exercise testing (CPX). A detailed clinical history was obtained and clinical examination performed. Exclusion criteria were as listed in the methods chapter (Chapter 2, page 55). Venous blood samples were taken at the time of recruitment to assay serum creatinine, urea, haemoglobin, serum calcium, inorganic phosphate and parathyroid hormone. Urine samples were assayed for urine protein-creatinine ratio. Estimated glomerular filtration rate (eGFR) was calculated using the 4-variable modification of diet in renal disease MDRD formula.[11]

Cardiopulmonary exercise tests (CPX)

Peak cardiac power output ($CPO_{max}$) was determined noninvasively during maximal cardiopulmonary exercise (CPX) testing. Full methodological details have been described in the previous chapter.[161] In summary, the subjects had baseline measurements for $O_2$ consumption, $CO_2$ production and resting cardiac output. Resting cardiac power output was calculated.[173,200] Subjects were then exercised on a

treadmill according to a standard Bruce protocol, until the subjects reached volitional exhaustion. Throughout the treadmill test, $O_2$ consumption, $CO_2$ production, end-tidal partial pressure of $CO_2$, tidal ventilation, and respiratory rate were measured using breath-to-breath analysis. Ventilatory ('anaerobic') threshold was measured by V-slope method.[179] After resting at least 40 min, a second treadmill test was performed to obtain maximum workload. The speed and incline of the treadmill were increased or decreased to allow each subject to sustain exercise for at least 5 min. During this exercise, the subjects needed to sustain a rate of $O_2$ consumption of at least 95% of that achieved in the initial treadmill exercise. Two or three cardiac output measurements were made using the Defares $CO_2$ rebreathing method.[174] The mean arterial blood pressure (MAP, mmHg) was calculated as MAP=DBP+0.412(SBP–DBP), where DBP is diastolic blood pressure and SBP is systolic blood pressure.[184] Cardiac power output (CPO), expressed in watt (W), was calculated [149]

3.2.1 Statistical analysis

$CPO_{max}$ was the primary outcome measure. In order to correct for the age difference, $CPO_{max}$ is also presented as a percentage of predicted value for age based on previous studies on healthy volunteers in the unit.[161] Comparison of $CPO_{max}$ among the study groups was performed using independent sample t-tests. Variations in $CPO_{max}$ and other study parameters across the study groups were analyzed by ANOVA. The association between $CPO_{max}$ and biochemical variables was assessed by Pearson's correlation. The haemodynamic determinants of cardiac reserve were evaluated using step-wise multiple regression analysis. Results are presented as mean±SD. $P<0.05$ was considered statistically significant.

## 3.3 Results

### 3.3.1 Subject characteristics

Anthropometric and biochemical characteristics of the study subjects are listed in Table 3.1. There were 70 male CKD patients with a mean age of 48.4±12.6 years covering the spectrum of CKD from stages 2 to 5 (CKD 2-5, pre-dialysis). The mean eGFR was 33.9±23.5 ml/min (CKD 5: 12.3±2.3 ml/min, CKD 4: 22.2±3.9, CKD 3: 43.2±7.9, CKD 2: 73.5±7.9 ml/min). The etiologies of CKD were as follows (number of patients in brackets): IgA nephropathy (19), polycystic kidney disease (15), reflux nephropathy & chronic pyelonephritis (15), membranoproliferative glomerulonephritis (3), Alport's nephropathy (2), interstitial nephritis (1), hypertensive nephropathy (1), focal segmental glomerulosclerosis (3), minimal change disease (1) and uncertain etiology (10). None of the patients had a history of primary cardiac disease (ischaemic, arrhythmic or valvular) or diabetes mellitus. Patients had no cardiac symptoms and thus all were in New York Heart Association (NYHA) class I. No patient had electrocardiographic evidence or symptoms of angina pectoris, myocardial ischaemia or arrhythmia during exercise testing. None had uncontrolled hypertension (mean resting SBP was 113.9±12.5 and DBP was 72.3±8.1 mmHg). Of the 70 patients, 61.4% were receiving angiotensin converting enzyme inhibitors (ACE-I), 34.8% angiotensin receptor antagonists and 20.0% β-adrenoceptor antagonists. The average numbers of anti-hypertensive agents taken per patient in CKD 2-3a, CKD 3b-4 and CKD 5 were 1.2, 1.6 and 1.7 respectively. For comparison, Table 3.1 also shows data from age- and sex-matched contemporaneous patients with confirmed heart failure (HF) in functional NYHA classes II & III recruited from cardiology outpatient clinics (n=25), who served as positive controls.

**Table 3.1**: Body composition and biochemical characteristics of study subjects

| | **Control (n=101)** | **CKD 2-3a (n=21)** | **CKD 3b-4 (n=27)** | **CKD 5 (n=22)** | **P** | **HF (n=25)** |
|---|---|---|---|---|---|---|
| **Age (years)** | 43.2±18.1 | 42.5±11.0 | 49.4±11.5 | 52.9±13.6 | <0.05 | 49.4±14.6 |
| **BMI ($kg/m^2$)** | 26.0±3.1 | 27.5±3.8 | 27.6±4.0 | 28.4±3.9 | NS | 25.1±3.2 |
| **BSA ($m^2$)** | 1.9±0.1 | 2.07±0.15 | 1.99±0.18 | 2.03±0.17 | NS | 1.93±0.18 |
| | | | | | | |
| **eGFR (ml/min)** | -- | 65.8±13.1 | 26.5±7.8 | 12.3±2.3 | <0.05 | 69.3±16.9 |
| **Creatinine (µmol/l)** | -- | 114.9±18.6 | 254.9±67.9 | 504.5±174.5 | <0.05 | 110.4±22.8 |
| **Urea (mmol/l)** | -- | 8.3±2.4 | 17.9±5.0 | 25.9±7.1 | <0.05 | 7.9±2.0 |
| **Haemoglobin (g/dl)** | -- | 15.0±1.2 | 13.2±1.5 | 11.9±1.2 | <0.05 | 14.4±1.13 |
| **Calcium (mmol/l)** | -- | 2.34±0.08 | 2.34±0.11 | 2.29±0.17 | NS | -- |
| **Phosphate (mmol/l)** | -- | 1.08±0.18 | 1.22±0.15 | 1.53±0.55 | <0.05 | -- |
| **Bicarbonate (mmol/l)** | -- | 27.24±3.02 | 23.74±3.15 | 21.32±2.97 | <0.05 | -- |
| **PTH (pmol/l)** | -- | 12.28±24.17 | 20.81±15.23 | 43.18±30.39 | <0.05 | -- |
| **Urine PCR (mg/mmol)** | -- | 25.99±28.9 | 96.44±112.52 | 130.94±140.94 | <0.05 | -- |

BMI: body mass index, BSA: body surface area, CKD: chronic kidney disease, HF: heart failure, PTH: parathyroid hormone, PCR: protein creatinine ratio. P value is for ANOVA across control and CKD groups.

### 3.3.2 Cardiopulmonary exercise test parameters

All CKD patients successfully performed cardiopulmonary exercise test (CPX) to volitional exhaustion with a mean duration of 10.5±2.9 min (Bruce protocol equivalent), mean peak respiratory exchange ratio (RER) of 1.16±0.09, a peak end tidal $pCO_2$ ($ETpCO_2$) of 37.2±5.9 mmHg, and maximal aerobic capacity ($VO_{2max}$) was 2.51±0.53 l/min (equivalent to 91.1±17.0% of average controls). The HF patients exercised for 5.5±2.8 min (Bruce protocol equivalent, $P<10^{-6}$ vs CKD) with a mean RER of 1.1±0.2, $ETpCO_2$ of 29.9±8.7 mmHg, and $VO_{2max}$ was 1.54±0.38 l/min (equivalent to 57.5±15.2% of average age-matched controls, $P<10^{-6}$ vs CKD).

#### 3.3.2.1 Resting haemodynamics

There were no differences between the resting haemodynamic parameters such as heart rate, blood pressure and stroke volume across different stages of CKD (Table 3.2 & Fig 3.1), whereas the resting cardiac output and resting cardiac power output showed a reducing trend with increasing severity of CKD. In contrast, the resting systemic vascular resistance (SVR) showed an increasing trend with increasing severity of CKD with a resting SVR in CKD 5 comparable to that of heart failure.

**Table 3.2:** Resting cardiopulmonary exercise parameters of study subjects

| Variables | Control (n=101) | CKD 2-3a (n=21) | CKD 3b-4 (n=27) | CKD 5 (n=22) | HF (n=25) |
|---|---|---|---|---|---|
| **$HR_{rest}$ beats/min** | 65.7±10.6 | 76.7±14.3* | 76.7±14.2* | 76.0±11.8* | 78.5±16.4* |
| **$SBP_{rest}$ (mmHg)** | 123.3±11.7 | 110.5±11.2 | 114.4±13.1 | 116.5±12.6 | 100.8±19.4* |
| **$DBP_{rest}$ (mmHg)** | 76.3±9.1 | 71.9±8.1 | 73.3±6.8 | 71.6±9.5 | 66.8±11.6* |
| **$MAP_{rest}$ (mmHg)** | 95.6±9.4 | 87.8±7.8* | 90.3±8.3* | 90.1±9.4* | 80.8±14.1* |
| **$CO_{rest}$ (l/min)** | 4.9±1.2 | 5.3±1.3 | 4.6±0.7 | 4.4±0.9 | 4.05±1.17* |
| **$SV_{rest}$ (ml/min)** | 75.5±20.6 | 71.1±14.3 | 63.5±13.7* | 60.8±13.1* | 56.3±21.1* |
| **$SVR_{rest}$ (dyn.sec.cm$^{-5}$)** | 1685.0± 511.9 | 1406.4± 347.8* | 1634.2± 346.1 | 1739.5± 450.5 | 1694.3± 435.3 |
| **$CPO_{rest}$ (W)** | 1.03±0.22 | 1.05±0.29 | 0.92±0.15* | 0.87±0.21* | 0.72±0.26* |

HR: heart rate, SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial pressure, CO: cardiac output, SV; stroke volume, SVR: systemic vascular resistance, $VO_{2max}$: peak oxygen consumption, $CPO_{max}$: peak cardiac power output. *P<0.05 vs Control on independent sample t-test.

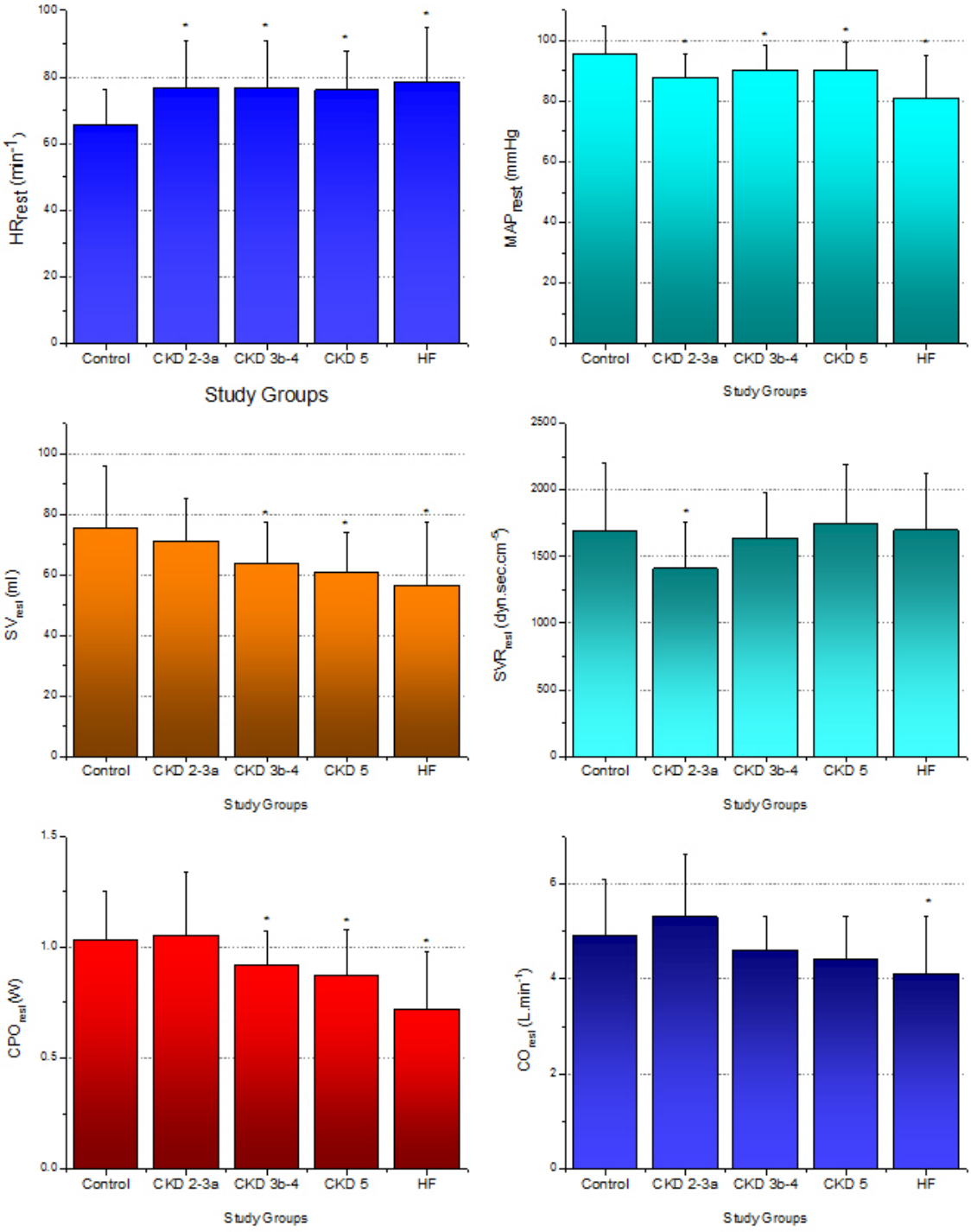


**Figure 3.1:** Resting cardiopulmonary exercise parameters of the study groups**.**
HR: heart rate, MAP: mean arterial pressure, CO: cardiac output, SV; stroke volume, SVR: systemic vascular resistance, $CPO_{max}$: peak cardiac power output, CKD: chronic kidney disease, HF: heart failure. *P <0.05 vs Control on independent sample t-test.

### 3.3.2.2 Peak haemodynamics

A summary of all peak haemodynamic parameters are presented in Table 3.3.

**Table 3.3** Peak cardiopulmonary exercise parameters of study subjects

| Variables | Control (n=101) | CKD 2-3a (n=21) | CKD 3b-4 (n=27) | CKD 5 (n=22) | HF (n=25) |
|---|---|---|---|---|---|
| **RER** | 1.21±0.1 | 1.15±0.09 | 1.15±0.11 | 1.16±0.06 | 1.09±0.29* |
| **Peak HR ($min^{-1}$)** | 172.9±17.4 | 171.3±19.7 | 158.7±17.9* | 148.7±24.9* | 128.9±40.1* |
| **Peak SBP (mmHg)** | 198.2±17.7 | 163.3±12.8* | 159.6±17.9* | 151.6±16.5* | 115.7±33.8* |
| **Peak MAP (mmHg)** | 119.5±11.6 | 114.3±8.5* | 111.8±10.6* | 104.7±10.5* | 87.8±22.1* |
| **Peak CO (l/min)** | 20.3±3.9 | 21.50±2.31 | 19.44±2.07* | 18.26±2.60* | 12.52±2.37* |
| **Peak SV (ml/min)** | 117.7±18.2 | 132.4±17.9* | 128.6±19.9* | 129.2±24.5* | 105.1±37.0* |
| **Peak SVR ($dyn.sec.cm^{-5}$)** | 492.2±118.8 | 430.6±60.1 | 464.1±77.1 | 465.9±67.6 | 593.2±237.6* |
| **$VO_{2max}$ (l/min)** | 2.98±0.9 | 2.91±0.46 | 2.48±0.43* | 2.17±0.43* | 1.54±0.38* |
| **% $VO_{2max}$** | 100.0±17.9 | 98.1±16.7 | 91.3±16.9* | 84.0±15.2* | 57.5±15.2* |
| **$CPO_{max}$ (W)** | 5.35±0.9 | 5.02±0.78 | 4.59±0.53* | 4.02±0.73* | 2.34±0.63* |
| **% $CPO_{max}$** | 100.0±15.5 | 95.1±13.0 | 88.2±10.1* | 78.5±13.9* | 44.8±11.3* |
| **Cardiac reserve ΔCPO (W)** | 4.34±0.89 | 3.98±0.82 | 3.66±0.5* | 3.15±0.68 | 1.62±0.6* |

HR: heart rate, SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial pressure, CO: cardiac output, SV; stroke volume, RER: respiratory exchange ratio, SVR: systemic vascular resistance, $VO_{2max}$: peak oxygen consumption, $CPO_{max}$: peak cardiac power output. *P<0.05 vs Control on independent sample t-test.

Peak cardiac power output ($CPO_{max}$) is impaired in CKD

CKD patients showed graded decline in $CPO_{max}$ with increasing severity of CKD (Table 3.3 & Fig 3.2). The $CPO_{max}$ of the study groups were 5.35±0.9 W in control, 5.02±0.78 W in CKD 2-3a, 4.59±0.53 W in CKD 3b-4 and 4.02±0.73 W in CKD 5 and 2.34±0.63 W in HF. The variation in $CPO_{max}$ across CKD groups was statistically significant (ANOVA $P<0.05$). Between groups, $CPO_{max}$ in CKD 5 was significantly impaired compared to both CKD 3b-4 ($P=0.003$) and CKD 2-3a ($P<10^{-4}$) but not as impaired as HF ($P<10^{-4}$). $CPO_{max}$ in CKD 3b-4 was significantly impaired compared to CKD 2-3a ($p=0.026$). In addition, the results are presented as percentages of the $CPO_{max}$ values predicted for age. The results were CKD 2-3a 95.1±13.0%, CKD 3b-4 88.2±10.1%, CKD 5 78.5±13.9% and in HF 44.8±11.3% (Figure 3.3). The cardiac functional reserve (ΔCPO) also showed graded reduction across the study groups (Table 3.3 & Figure 3.4).

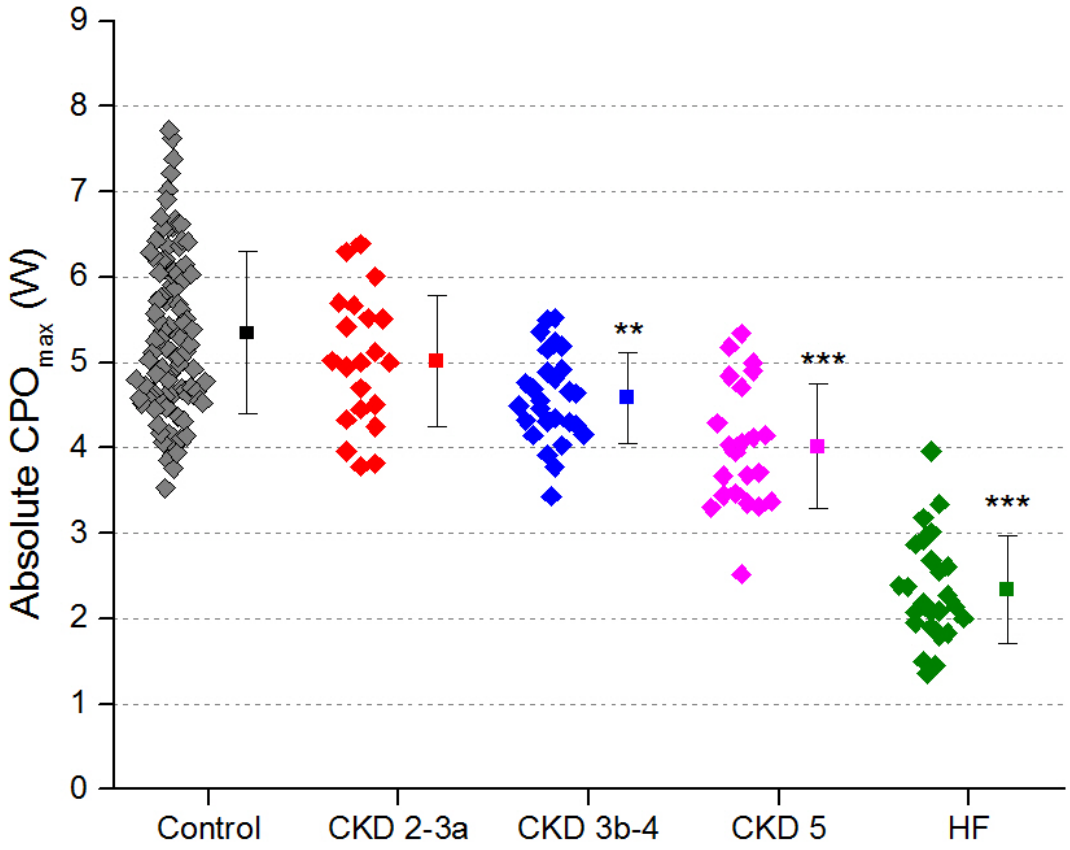


**Figure 3.2:** Graded reduction in absolute $CPO_{max}$ across the study groups.
CKD: chronic kidney disease, HF: heart failure. **P<$10^{-3}$, *** P<$10^{-6}$ vs Control on independent sample t-test.

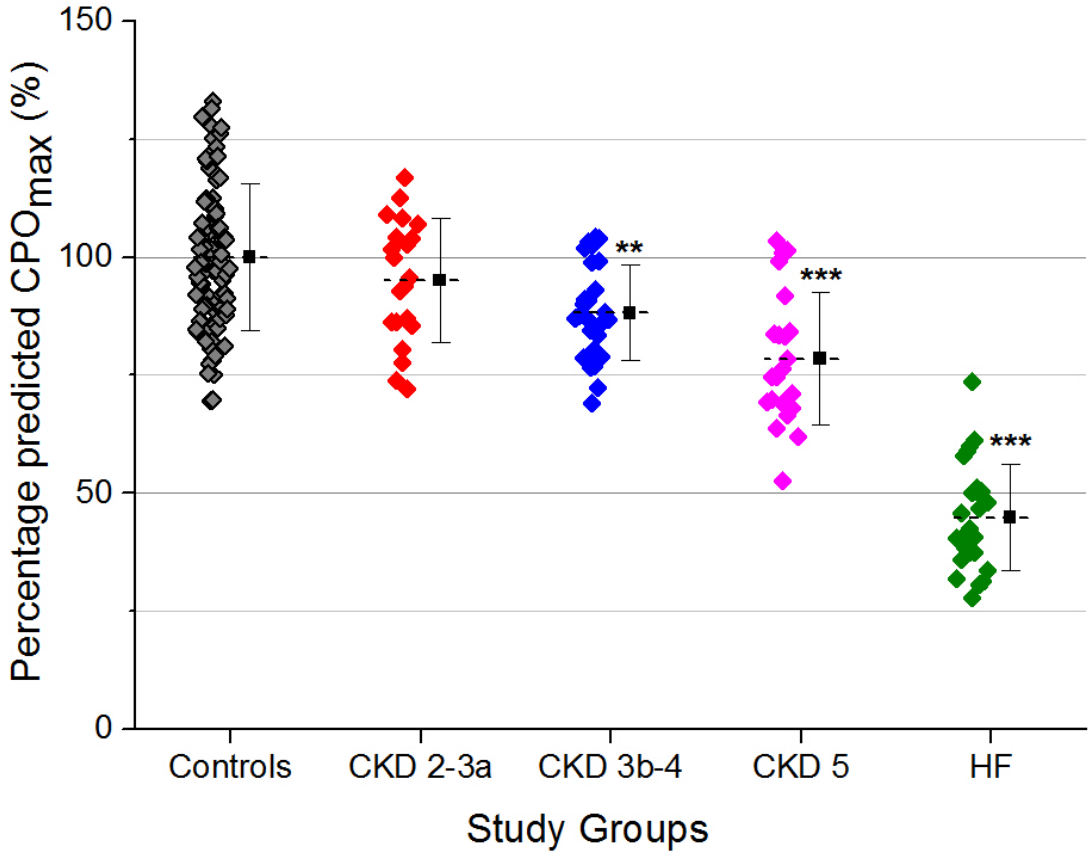


**Figure 3.3:** Graded reduction in percentage $CPO_{max}$ (age-corrected) across the study groups. CKD: chronic kidney disease, HF: heart failure. **$P<10^{-3}$, *** $P<10^{-6}$ vs Control on independent sample t-test.

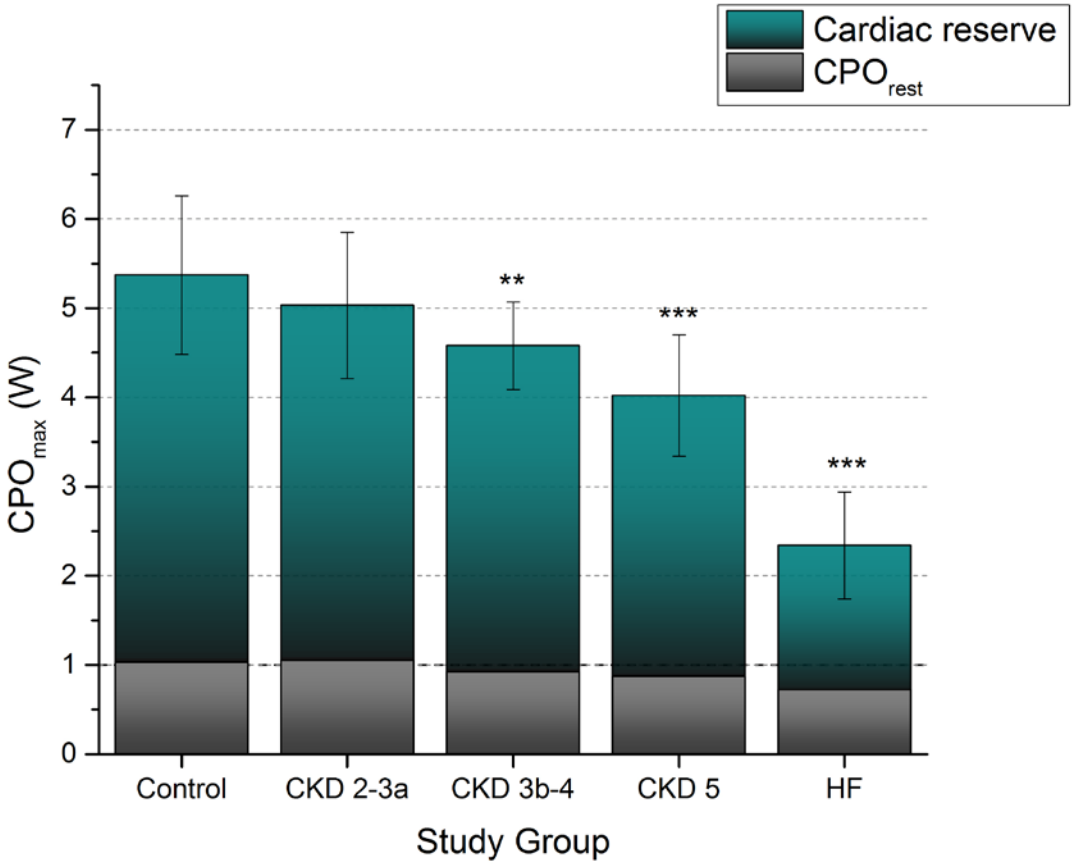


**Figure 3.4:** Cardiac reserve (ΔCPO) of study groups. CKD: chronic kidney disease, HF: heart failure, CPO cardiac power output. **P<$10^{-3}$ for cardiac reserve vs Control, ***P>$10^{-6}$ for cardiac reserve vs Control.

Peak cardiac output and peak mean arterial pressure are impaired in different stages of CKD

Figure 3.5 shows the differences in peak cardiac output (CO) and peak mean arterial pressure (MAP), the flow and pressure generating capacities of the heart respectively, across the study groups. Peak CO and peak MAP were significantly lower in CKD 5 compared to CKD 2-3a and CKD 3b-4 ($P<0.05$). However, the values were not as impaired as in HF ($P<0.05$).

Peak heart rate and heart rate reserve are impaired in CKD

The peak heart rate was significantly impaired in CKD 5 compared to CKD 2-3a and CKD 3b-4 (both $P<0.05$) but not as impaired as in HF ($P<0.05$) (Table 3.3). The heart rate reserve (HRR), measured as the difference between peak and resting heart rate across the study groups were CKD 2-3a 94.6±12.9, CKD 3b-4 82.1±15.4, CKD 5 72.7±18.4 and HF 55.8±24.1 beats/min. The HRR in CKD 5 was significantly lower compared to other CKD groups ($P<0.05$). In addition, the age adjusted HRR is significantly lower in CKD 5 compared to other CKD groups ($P<0.05$) but not as impaired as in HF ($P<0.05$) (Figure 3.6).

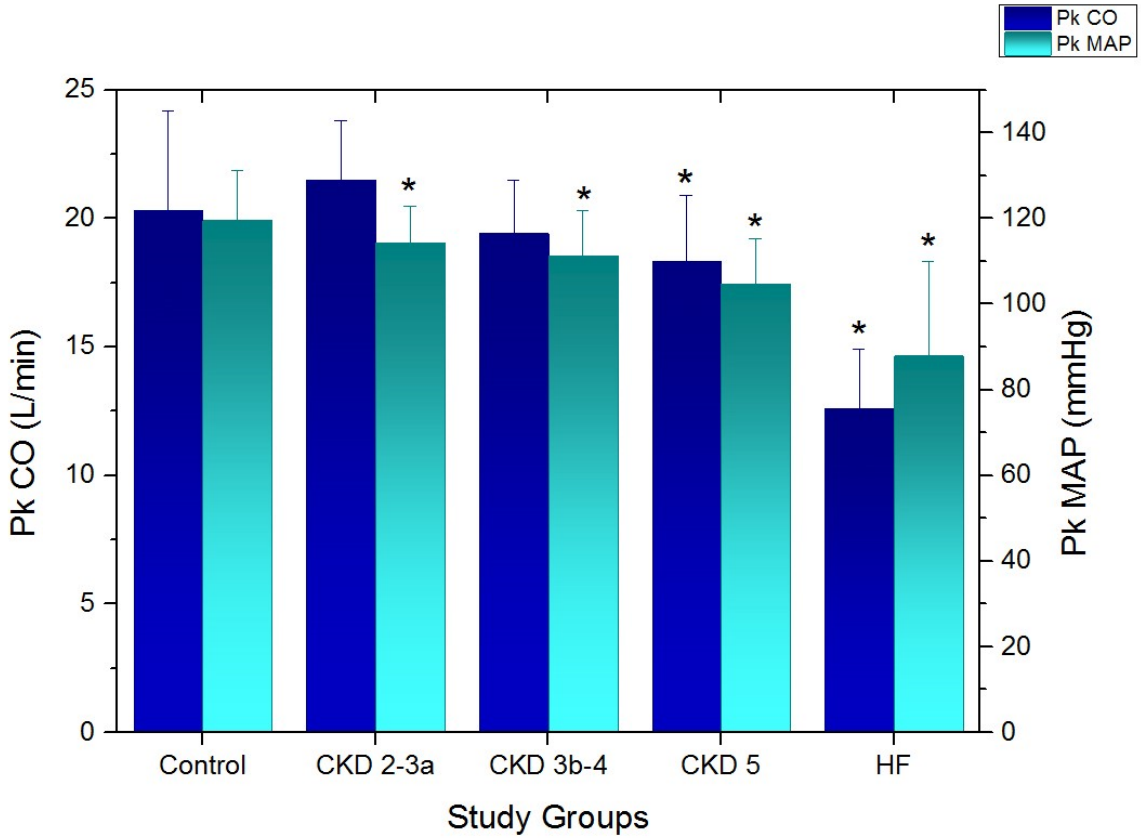


**Figure 3.5:** Peak cardiac output (Pk CO) and peak mean arterial pressure (Pk MAP) across the study groups. CKD: chronic kidney disease, HF: heart failure.
*P<0.05 vs Control on independent sample t-test

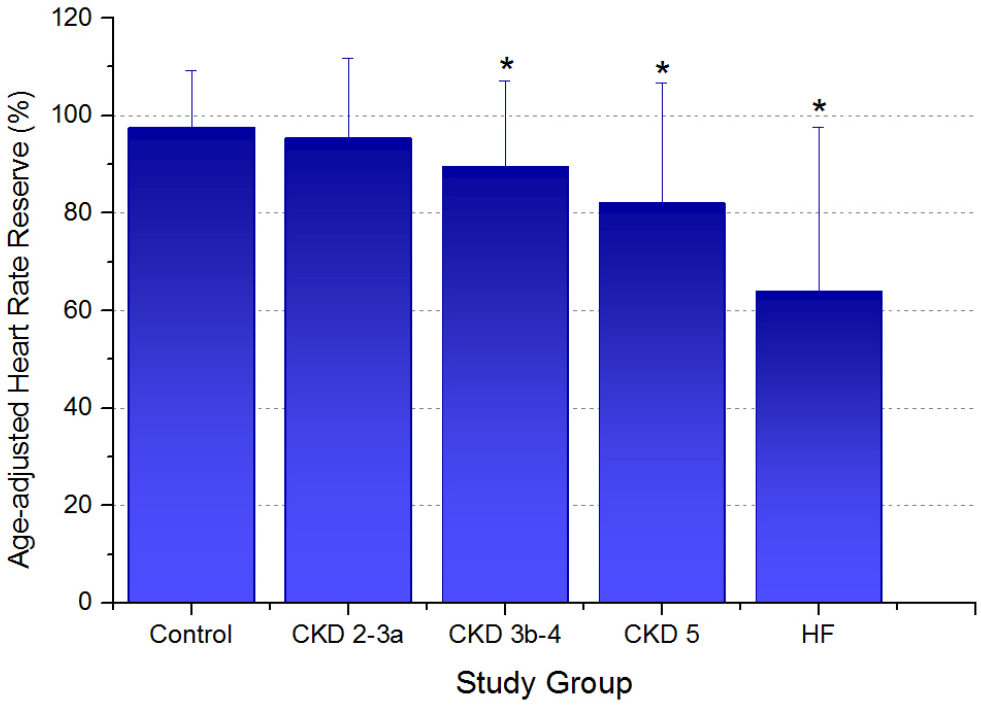


**Figure 3.6:** Age adjusted heart rate reserve in percentage across the study groups. CKD: chronic kidney disease, HF: heart failure. *P<0.05 vs Control on independent sample t-test.

Increase in stroke volume partially offsets reduced chronotropic reserve in CKD

The components of peak cardiac output, the peak stroke volume and the peak heart rate are shown in Figure 3.7. The peak stroke volume (SV) in CKD stages were higher compared to control. The effect of impaired chronotropic reserve on cardiac output was partially offset by this increase in stroke volume.

There is no difference in peak systemic vascular resistance across CKD groups

Although the resting systemic vascular resistance (SVR) showed an increasing trend with increasing severity of CKD (Table 3.2), the peak SVR was comparable between CKD groups. The peak SVR of heart failure patients remained higher than all stages of CKD and control (all $P<0.05$) (Table 3.3).

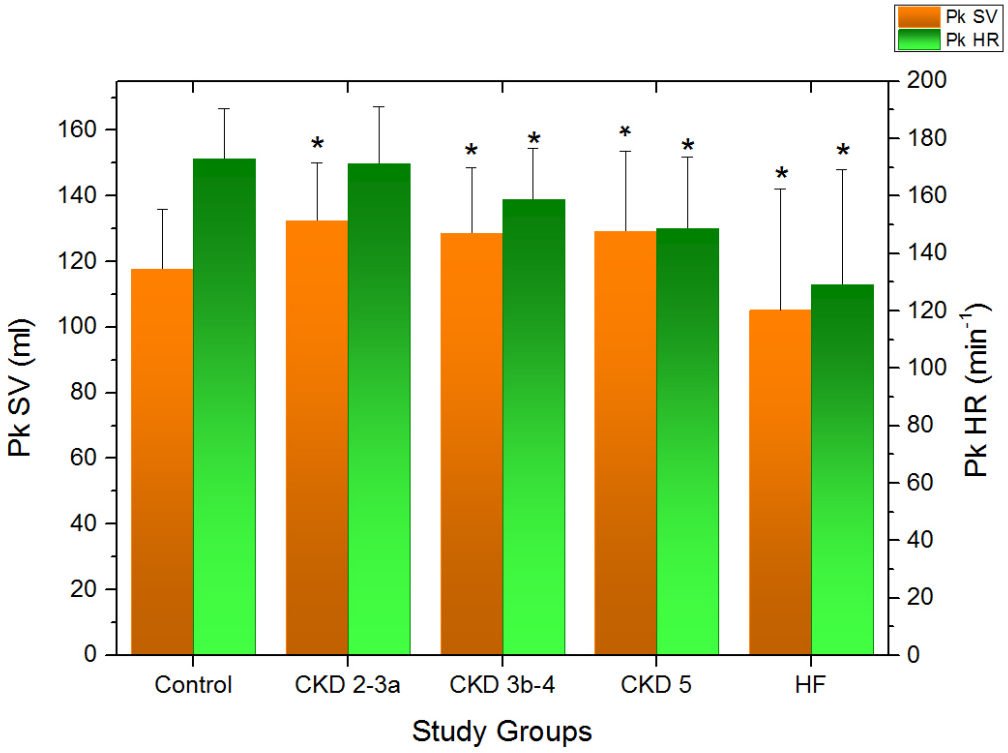


**Figure 3.7:** Peak stroke volume (Pk SV) and peak heart rate (Pk HR) across the study groups. CKD: chronic kidney disease, HF: heart failure. *P<0.05 vs Control on independent sample t-test.

### 3.3.2.3 Determinants of cardiac reserve in CKD

The independent predictors of cardiac reserve (ΔCPO) in CKD were identified on a stepwise multiple regression analysis. The increment in the pressure generating capacity of the heart (ΔMAP, the difference between peak and resting MAP) was the strongest independent predictor ($\beta=0.51$, $P<10^{-4}$) followed by the increment in the flow generating capacity (ΔSV, the difference between peak and resting stroke volume) ($\beta=0.49$, $P<10^{-4}$) and the chronotropic reserve (ΔHR) ($\beta=0.42$, $P<10^{-4}$). The other independent predictors were eGFR ($\beta=0.23$, $P=0.003$) and BMI ($\beta=0.15$, $P=0.03$). No independent association was demonstrated with haemoglobin (Hb) or systemic vasodilatory capacity (ΔSVR).

### 3.3.2.4 Association between $CPO_{max}$ and CKD related biochemical parameters

$CPO_{max}$ had significant positive correlation with eGFR, haemoglobin and serum bicarbonate, and significant negative correlation with urea, parathyroid hormone and inorganic phosphate. No correlation existed with serum calcium and urine protein creatinine ratio (Figure 3.8). Independent association of individual biochemical variables with $CPO_{max}$ could not be assessed because of strong co-linearity among the biochemical parameters.

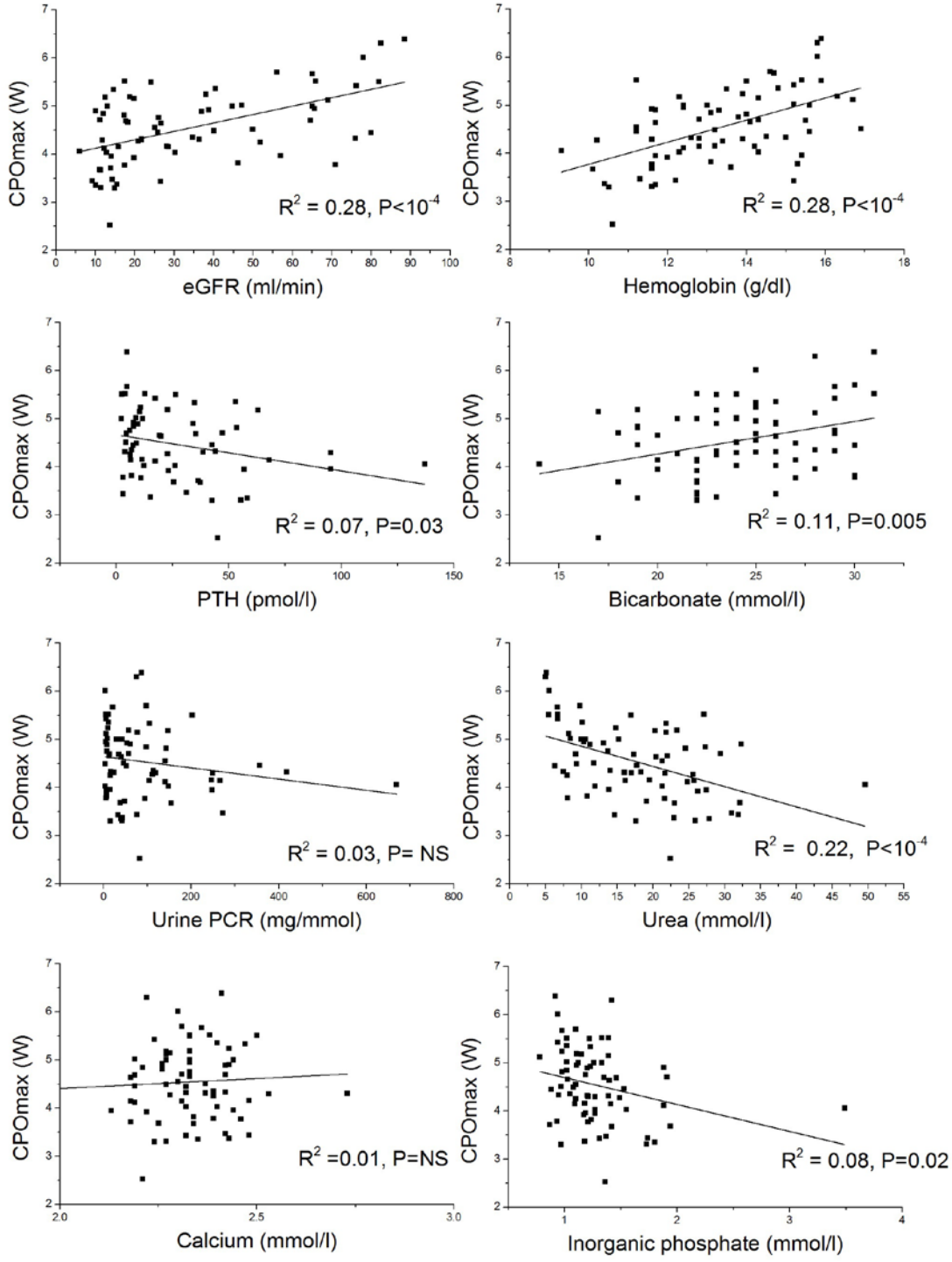


**Figure 3.8:** Association between $CPO_{max}$ and CKD-related biochemical parameters on Pearson's correlation. PTH: parathyroid hormone.

### 3.3.2.5 Relationship between maximal aerobic capacity, peak cardiac output and peripheral $O_2$ extraction

Simultaneous measurements of $CPO_{max}$ and $VO_{2max}$ in patients with CKD allowed evaluation of the determinants of these parameters in CKD. Furthermore, simultaneous measurements of $VO_2$ and cardiac output at rest and peak exercise enabled calculation of arterio-venous difference in $O_2$ concentration [$C(a\text{-}v)O_2$] at rest and peak exercise for the first time in CKD patients using Fick's equation $VO_2 = CO \times C(a\text{-}v)O_2$. The differential role of central (cardiac output) and peripheral [$C(a\text{-}v)O_2$] determinants of maximal aerobic capacity is shown in Figure 3.9. Whereas in HF the reduction in $VO_{2max}$ was mediated primarily through reduction in cardiac output, in CKD impaired $C(a\text{-}v)O_2$ played a significant role. Indeed, on a step-wise multiple regression analysis $\Delta C(a\text{-}v)O_2$ was the strongest predictor of the $VO_2$ reserve ($VO_{2max} - VO_{2rest}$) with a $\beta$ value of 0.53 ($P<10^{-6}$). The other independent predictors of $VO_2$ reserve were ($\beta$ and P values in brackets) $\Delta SV$ ($\beta=0.32$, $P=10^{-3}$), $\Delta HR$ ($\beta=0.28$, $P=0.002$), Hb ($\beta=0.31$, $P=10^{-3}$), age ($\beta=-0.28$, $P=10^{-3}$) and BMI ($\beta=0.2$, $P=0.003$). $C(a\text{-}v)O_2$ showed strong correlation with $VO_{2max}$ ($r=0.783$, $P<10^{-6}$) and haemoglobin level ($r=0.41$, $P<0.001$) in CKD.

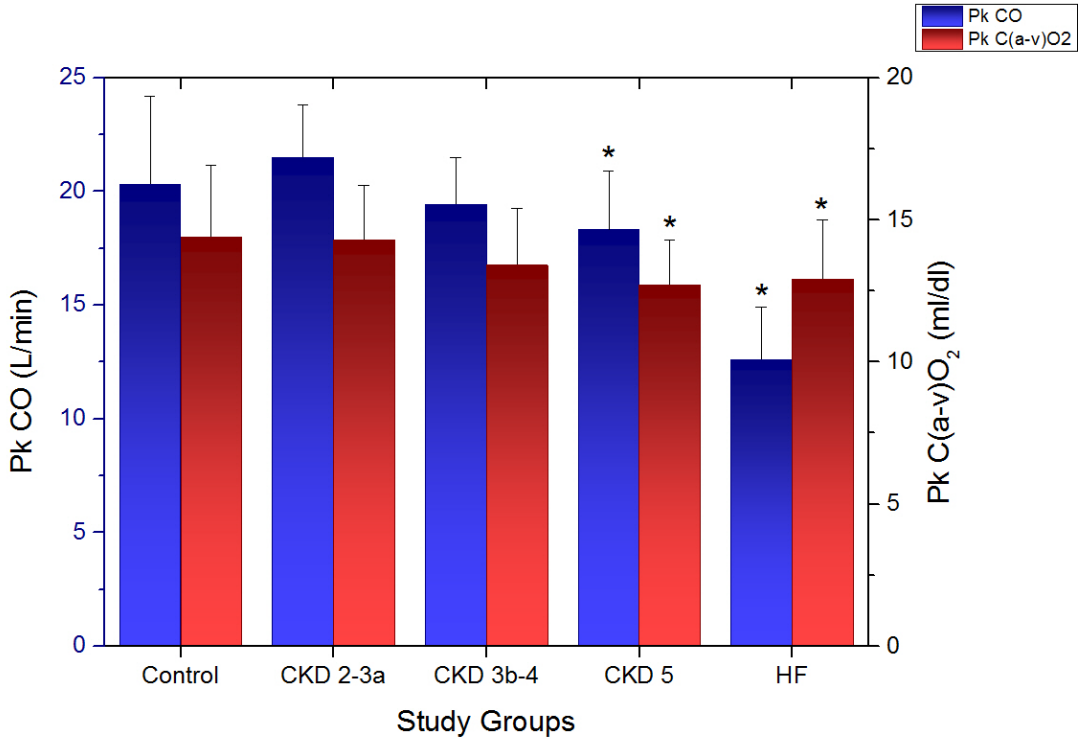


**Figure 3.9**: Differential changes in peak cardiac output (Pk CO) and peak peripheral $O_2$ extraction C(a-v)$O_2$ across the study groups. Aerobic capacity in CKD is reduced as a result of reduction in both cardiac output and C(a-v)$O_2$ unlike in heart failure where reduction in cardiac output is the predominant factor. CKD: chronic kidney disease, HF: heart failure. *P<0.05 vs Control on independent sample t-test.

**Effect of beta blockers on central haemodynamics in CKD patients**

Linear regression was performed, controlling for eGFR, to compare haemodynamic parameters between CKD patients who were on beta blockers and those who were not. Results showed that there were no significant differences in peak CPO, peak CO and peak MBP. The mean peak CPO was lower in the beta blocker group by 0.03 W (P= 0.96), the mean peak CO was lower by 0.13 l/min (P=0.54) and the mean peak MBP was lower by 3.72 mmHg (P=0.23). However, significant differences were present between the mean peak HR and mean peak SV between the groups. The mean peak HR was lower in the beta blocker group by 26.85 $min^{-1}$ ($P<10^{-3}$), whereas the mean peak SV was greater in the beta blocker group by 26.63 ml ($P<10^{-3}$) offsetting the reduction in peak HR.

## 3.4 Discussion

In the present study, I tested the hypothesis that peak cardiac power and cardiac functional reserve are impaired in asymptomatic chronic kidney disease patients in the absence of any known cardiac diseases or diabetes mellitus, and the study results support the hypothesis. Furthermore, the peak cardiac performance was shown to be diminished proportional to CKD severity. As asymptomatic, non-diabetic CKD patients with no overt cardiac disease were studied, the results show that CKD *per se* is associated with cardiac dysfunction. This dysfunction is only revealed under conditions of peak exercise, suggesting that in CKD patients, like conventional cardiac failure, compensatory mechanisms maintain resting cardiac performance within normal limits. Although cardiac structural changes have been studied in the past in asymptomatic CKD patients, the present study has demonstrated the functional impairment for the first time in such patients.

The central haemodynamic alterations underlying the impaired peak cardiac performance in CKD mirror the changes in symptomatic heart failure patients albeit of lesser magnitude. The impairment in cardiac power results from impairment in both flow (peak CO) and pressure (peak MAP) generating capacities of the heart akin to HF. Further analysis of the components of cardiac output such as heart rate and stroke volume reveal impaired chronotropic reserve in CKD not unlike HF. Although the stroke volume appears to be preserved in CKD, a parameter that incorporates both volume and pressure generating capacities and is afterload-independent such as cardiac power reveals the underlying impairment in cardiac contractility in CKD. In summary, the central haemodynamic changes seen in symptomatic heart failure appears to be evolving in asymptomatic CKD patients even in the absence of any primary cardiac disease.

Heart failure and CKD share several common pathophysiological mediators with the potential to cause progressive myocardial dysfunction. These include renin angiotensin aldosterone system (RAAS) activation, sympathetic activation, pro-inflammatory cytokines and myocardial wall stress.[84,201-205] In heart failure, these mediators were shown to cause direct cardiomyocyte toxicity by inducing apoptosis and necrosis, and pathological hypertrophy resulting in progressive myocardial dysfunction.[115,118,206-209] The existence of the above mediators in the uraemic milieu raises the possibility of similar mechanism of myocardial dysfunction in CKD.

The above similarities between CKD and HF in the biomechanics of myocardial dysfunction and the mediators of such dysfunction draw our attention to potential therapies to attenuate or reverse the process. Whereas in heart failure the benefits of RAAS blockade and beta blockade in preventing the progression of myocardial dysfunction has been shown consistently,[210-212] the evidence for such treatments in uraemic cardiomyopathy is still lacking.

It is interesting to note that the novel finding of the study could potentially explain some of the diverse phenomena shown to be associated with CKD in the past. It has been shown that CKD is associated with high mortality at times of stress such as surgery [213] or sepsis. [162] Diminished cardiac reserve in CKD could provide an explanation for this phenomenon. The study implies that a superimposed ischaemic event on a uraemic heart, with poor functional reserve, would potentially have a devastating effect. This has in fact been demonstrated in a large observational study on dialysis patients that reported an astounding 50% mortality at one year and 90% mortality at 5 years after a myocardial infarction.[163] It is a well-established fact that renal dysfunction confers a serious adverse prognosis in heart failure.[26] It has been shown that renal dysfunction is more than just a marker of severity but is an

independent risk factor of HF mortality.[27] The finding, that renal dysfunction per se could cause impaired cardiac functional reserve, offers a possible pathophysiological explanation for the negative impact of renal dysfunction on heart failure survival.

I utilised physical exercise to drive the heart to its peak performance because it is a physiological stimulus as opposed to pharmacological stimulus such as dobutamine. As CPX is the study tool, it is natural to assume $CPO_{max}$ as being similar to conventional CPX parameter such as peak $O_2$ consumption ($VO_{2max}$). However, head-to-head comparison between $VO_{2max}$ and $CPO_{max}$ in heart failure studies have demonstrated that $CPO_{max}$ is a direct indicator of cardiac dysfunction and the best predictor of survival in patients with failing heart.[153,154] Some of the CPX studies in end stage renal disease have suggested that conventional CPX parameters could serve as a measure of cardiac reserve in this population.[158] The premise is that $VO_2$ is a product of cardiac output and arterio-venous difference in oxygen concentration [$VO_2$= CO x $C(a\text{-}v)O_2$] and hence $VO_2$ could serve as a surrogate of CO. This simplified model assumes that $C(a\text{-}v)O_2$ is a constant irrespective of patient group. However, as demonstrated in the present study, $C(a\text{-}v)O_2$ at peak exercise is reduced in CKD compared to healthy controls [Fig 3.9]. This impaired peripheral $O_2$ extraction is likely to be related to anaemia (which the study data support), plus possible uraemic skeletal myopathy, though further studies would be required to fully elucidate the role of central, peripheral and biochemical determinants of this phenomenon. Whatever the cause, the findings of the study indicate that $VO_{2max}$ would be a less reliable indicator of cardiac reserve in CKD patients.

Cardiac power output and cardiac reserve were not shown to be influenced by beta blockade therapy. The reduction in peak heart rate associated with beta blockade

was shown to be offset by the increase in SV and hence there was no net effect on the cardiac performance. Almost all CKD patients were treated with RAAS blockade, but if this has affected the results, the effect was uniform. Furthermore, as cardiac output and afterload have a linear inverse relationship,[214] and $CPO_{max}$ is an index that incorporates measures of both volume and pressure, the changes in arterial pressure are offset by changes in the volume generated. Hence $CPO_{max}$ remains afterload-independent akin to other pressure-volume indices such as stroke work, stroke work index etc.[214]

## 3.5 Limitations

As the first ever study exploring the impact of CKD directly on peak cardiac power, I utilised a cross-sectional study design. A longitudinal study design in the same patients as the disease progresses would be better suited to demonstrate a causal relationship between uraemia and cardiac dysfunction. However, most etiologies of CKD (especially in the absence of co-morbid CVD or DM) run an indolent course progressing over many years making such a study challenging to undertake. Alternatively, a longitudinal study design testing intervention such as kidney transplantation or novel dialysis strategies would be highly pertinent in the future.

The absence of data on renal function of healthy volunteers is a limitation. However, as the prevalence of severe CKD (CKD 4 and 5) in general population is less than 1%,[15,215] the likelihood of undiagnosed severe CKD among the healthy controls is very minimal.

A criticism of employing CPX in the evaluation of cardiac performance is that inadequate patient effort could be a confounder. In our study, the respiratory exchange ratio (RER) of the CKD patient in all 3 groups were > 1.1 demonstrating good exercise effort ensuring that the impairment in cardiac functional reserve is not

attributable to inadequate effort. The operator measuring CPX parameters and blood pressure is not blinded to the severity of CKD and I recognise this as a potential confounder.

The study has demonstrated that haemoglobin is not an independent predictor of $CPO_{max}$. Studies that simultaneously measured peak cardiac output and $VO_{2max}$ before and after erythropoietin therapy/haemodilution had also shown that altered haemoglobin affected $VO_{2max}$ measures but not peak cardiac output.[216] Though it must be acknowledged my evaluation is limited to statistical analysis of a cross sectional study. Further longitudinal studies in CKD may be worthwhile in the future to evaluate the effect of anaemia correction on central haemodynamics.

CKD patients suffer from metabolic acidosis. Although the effect of acidosis and alkalosis on the $O_2$ dissociation curve is well known, the effect on the $CO_2$ dissociation curve has not been well studied. Hence, it is not known whether metabolic acidosis in CKD affects the non-invasive cardiac output values obtained by $CO_2$ rebreathing method in CKD patients.

## 3.6 Conclusion

The study 1 of the thesis supports the hypothesis that cardiac function is impaired in asymptomatic CKD patients and the dysfunction appears to be associated with CKD alone in the absence of any known cardiovascular co-morbidities. In the next chapter, I describe the effect of renal transplantation on the subclinical cardiac dysfunction in CKD.

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# CHAPTER 4

# Study 2

# Reversal of subclinical cardiac dysfunction in asymptomatic chronic kidney disease patients following renal transplantation

## 4.1 Introduction

Uraemic cardiomyopathy (UCM), the characteristic heart disease of chronic kidney disease (CKD) progresses relentlessly with the progression of CKD. Starting dialysis does little to prevent the progression, let alone the reversal, of UCM.[9] The treatment modality that has consistently shown to improve cardiovascular disease (CVD) morbidity and mortality in end stage renal disease (ESRD) is renal transplantation (RTx).[217,218] The pathophysiological basis of such cardiovascular benefits are not fully understood. Cardiac imaging studies have shown that LVH, the characteristic structural abnormality of UCM, regresses with kidney transplantation.[219,220] However, elucidation of the corresponding cardiac functional changes are still lacking.

Previous echocardiographic studies on patients with impaired left ventricular ejection fraction (LVEF) pre-transplantation had shown improvement in (LVEF) post transplantation and the improvement had been variably attributed to correction of fluid status and anaemia.[220-222] However, it is not known whether such functional benefits are applicable to a broader group of transplant candidates who are mostly asymptomatic. In Study 1 of the thesis I demonstrated the presence of subclinical cardiac dysfunction in asymptomatic CKD patients without known cardiac diseases or diabetes. In the present chapter of the thesis I evaluated whether successful renal transplantation, with significant improvement in renal function, reversed this functional deficit.

## 4.2 Methodology

A detailed methodology is discussed in chapter 2 of the thesis. A brief methodology relevant to this chapter is presented here.

*Study design:* A prospective study of 6 asymptomatic male CKD patients (>18 years) before and after renal transplantation (RTx).

*Investigations:* CPX test was performed as described in the methodology section. The participants in Study 1 who received a RTx also underwent a second CPX test post RTx. The post-transplant CPX test was performed after at least 3 months to allow full recovery from the surgical procedure. In addition, blood samples were obtained to measure CKD-related biochemistry pre and post transplantation. Comparison between study parameters before and after transplantation was performed using paired sample t-test. A *P* value of <0.05 is considered significant. Results are presented as mean±SD.

## 4.3 Results

The patients had a mean age of 48.4 years. Their underlying aetiologies were IgA nephropathy (2 patients), polycystic kidney disease (2 patients), interstitial nephritis (1 patient) and reflux nephropathy (1 patient). Of the 6 patients, 3 were receiving ACE-i, 3 were receiving ARB and 4 were receiving beta blocker before RTx. Post RTx all patients were receiving Tacrolimus, 3 were receiving ACE-i/ARB and 1 was receiving a beta blocker. None of the patients were on corticosteroids.

The median time to CPX testing post-transplantation was 5 months (range: 3 to 11 months). Their eGFR improved from 12.5±4.0 mL/min before to 64.9±6.5 mL/min after transplantation (*P*=0.004).

The changes in CPX parameters before and after transplantation are presented in Table 4.1. Following transplantation all 6 patients showed increased $CPO_{max}$, with the overall mean $CPO_{max}$ rising by 19.1% from 3.82±1.03 to 4.55±0.80 W (*P*=0.003) (Fig 4.1). The improvement in $CPO_{max}$ resulted from increases in both peak mean arterial pressure (98.5±15.9 vs 110.8±12.3 mmHg, *P*=0.001) and peak cardiac output (17.29±2.37 vs 18.47±2.05 $L.min^{-1}$, *P*=0.04) (Fig 4.2 & 4.4). Transplantation was also associated with statistically significant improvement in peak exercise heart rate (134.8±26.1 vs 156.8±29.1 $min^{-1}$, *P*=0.04) (Fig 4.3). The cardiac reserve (ΔCPO) increased from 2.95±0.90 to 3.45±0.78 W (P=0.03). No statistically significant change was seen in $VO_{2max}$ (2.44±0.6 vs 2.62±0.45 L/min, *P*=NS) or haemoglobin (114.7±16.7 vs 127.8±10.5 g/L, *P*=NS).

Differential role of the components of $CPO_{max}$

The relative influence of change in peak cardiac output (peak CO) and change in peak mean arterial pressure (peak MAP) from the pre RTx to the post RTx state on the change in $CPO_{max}$ was analysed using multiple regression analysis. The results showed that improvement in peak CO was the strongest predictor of improvement in $CPO_{max}$ (β=0.74, P=0.005) vs peak MAP (β=0.39, P=0.028). The change in peak CO was equally influenced by the change in stroke volume (β=2.66, P=0.04) and change in heart rate (β=2.43, P=0.04).

**Table 4.1** Biochemical and CPX parameters before and after kidney transplantation

| | **Pre** | **Post** | **P value** |
|---|---|---|---|
| **Biochemistry** | | | |
| eGFR ($mL.min^{-1}$) | 12.5±4.0 | 64.9±6.5 | 0.00042 |
| Hb ($g.L^{-1}$) | 114.7±16.7 | 127.8±10.5 | 0.12 |
| **Resting CPX parameters** | | | |
| $HR_{rest}$ ($min^{-1}$) | 68.3±13.3 | 78.7±15 | 0.10 |
| MAP (mmHg) | 87.1±10.9 | 90.5±4.3 | 0.54 |
| $VO_2$ ($L.min^{-1}$) | 0.34±0.10 | 0.34±0.09 | 0.98 |
| CO ($L.min^{-1}$) | 4.16±0.98 | 5.35±1.19 | 0.03 |
| $CPO_{rest}$ (Watts) | 0.84±0.24 | 1.08±0.23 | 0.02 |
| **Peak CPX parameters** | | | |
| RER | 1.07±0.05 | 1.14±0.03 | 0.03 |
| $VO_{2max}$($L.min^{-1}$) | 2.44±0.60 | 2.62±0.45 | 0.21 |
| $VO_{2max}$/kg ($ml.min^{-1}.kg^{-1}$) | 27.5±5.5 | 29.3±2.5 | 0.48 |
| $HR_{max}$ ($min^{-1}$) | 134.8±26.1 | 156.8±29.1 | 0.04 |
| $MAP_{max}$ (mmHg) | 98.5±15.9 | 110.8±12.3 | 0.001 |
| $CO_{max}$ ($L.min^{-1}$) | 17.29±2.37 | 18.47±2.05 | 0.04 |
| $CPO_{max}$ (W) | 3.82±1.03 | 4.55±0.80 | 0.003 |
| ΔCPO (W) | 2.95±0.90 | 3.45±0.78 | 0.03 |

Hb: haemoglobin, eGFR: estimated glomerular filtration rate, CO: cardiac output, $CPO_{rest}$: resting cardiac power output, $CPO_{max}$: peak exercise cardiac power output, $HR_{rest}$: resting heart rate, $HR_{max}$: peak exercise heart rate, RER: respiratory exchange ratio at peak exercise, $VO_{2rest}$: resting $O_2$ consumption rate, $VO_{2max}$: peak $O_2$ consumption rate. P value is for paired sample t-test.

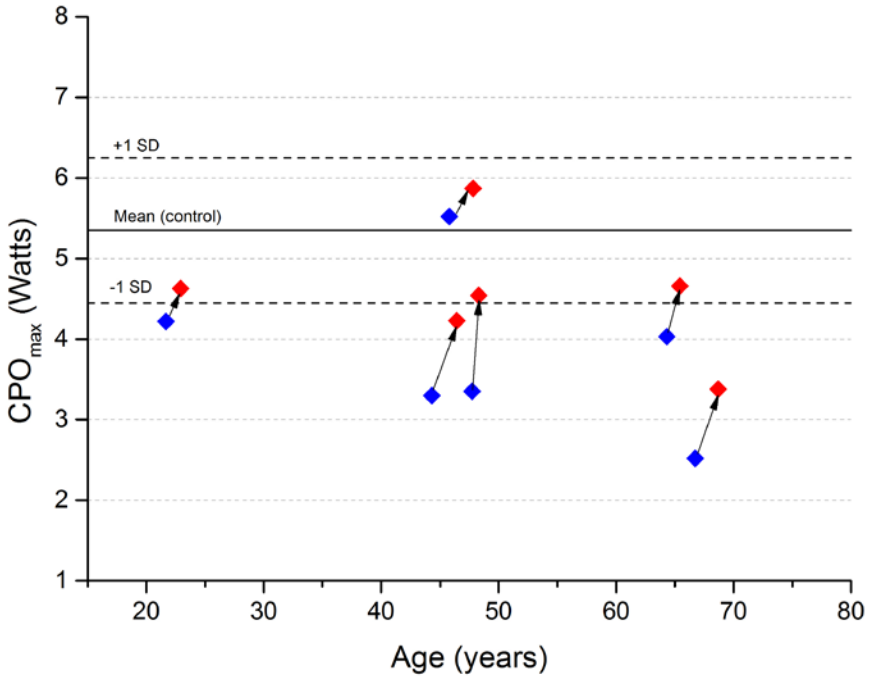


**Fig 4.1:** Peak cardiac power output ($CPO_{max}$) of CKD patients before (blue markers) and after (red markers) renal transplantation. All patients demonstrated improvement in $CPO_{max}$ with transplantation.

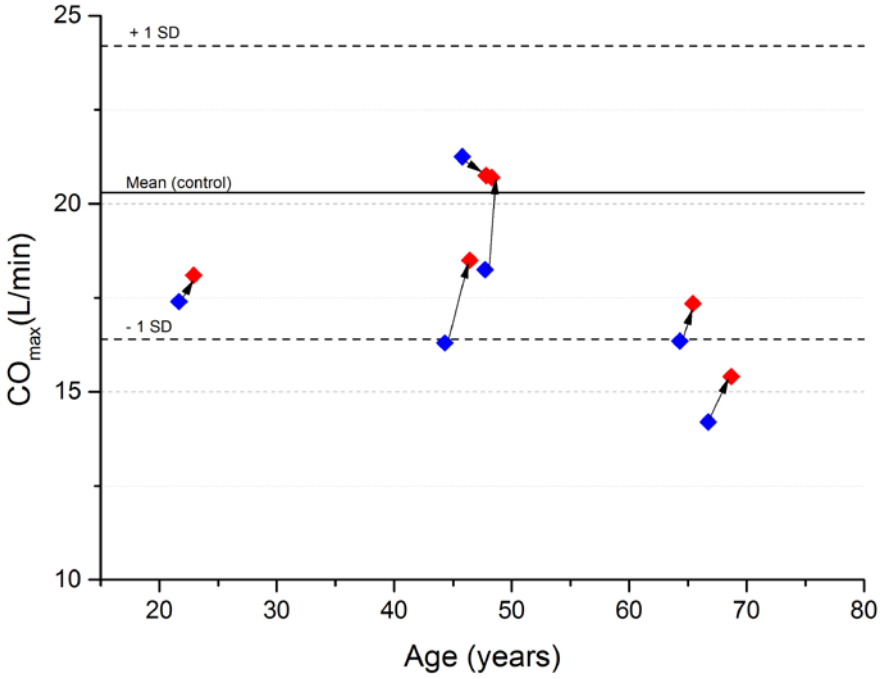


**Fig 4.2:** Peak cardiac output ($CO_{max}$) of CKD patients before (blue markers) and after (red markers) renal transplantation

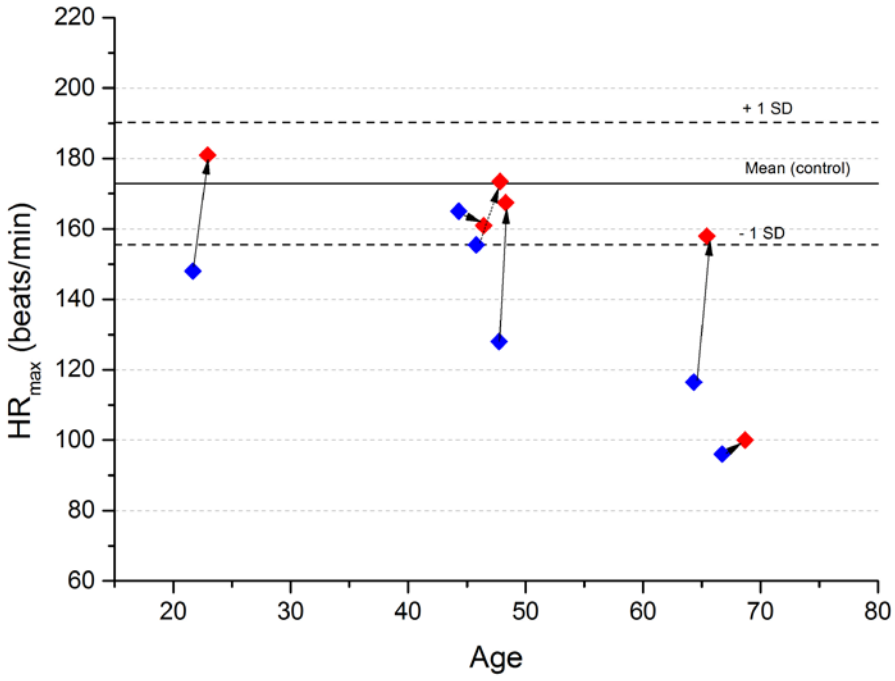


**Fig 4.3:** Peak heart rate ($HR_{max}$) of CKD patients before (blue markers) and after (red markers) renal transplantation.

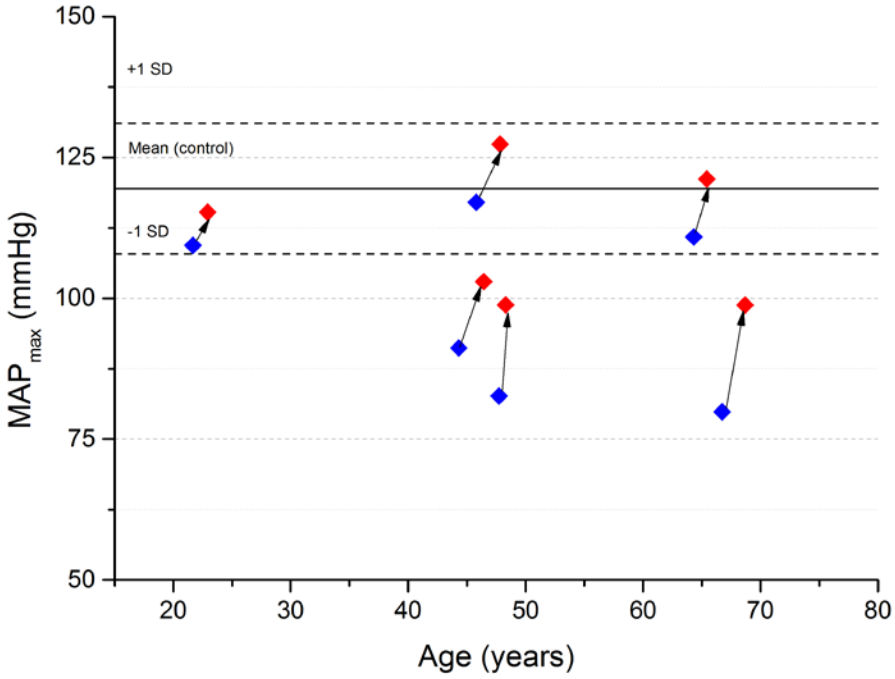


**Fig 4.4:** Peak mean arterial pressure ($MAP_{max}$) of CKD patients before (blue markers) and after (red markers) renal transplantation.

## 4.4 Discussion

The results demonstrate that *all patients* showed improvement in peak cardiac power and cardiac reserve post RTx. The haemodynamic alterations such as impaired pressure and volume generating capacities and the impaired chronotropic reserve, demonstrated in the Study 1 of the thesis, all appear to improve post transplantation. The results, by demonstrating the reversal of cardiac functional deficit, complement the available literature that demonstrated reversal of LVH, in supporting the hypothesis that renal transplantation aids cardiac *reverse remodelling* in UCM.

The beneficial cardiac effects of RTx are a possible consequence of alterations in the uraemic milieu brought about by RTx. Some of the uraemic factors that are potentially altered by RTx are presented below. However, further large studies are needed to fully elucidate the underlying mechanism of cardiac improvement post RTx.

- Correction of sodium and water retention: Renal transplantation has the potential improve the volume status. In a group of 32 ESRD patients who had LV dilatation and underwent RTx, the left ventricular volume fell from 116 ±3.1 ml/$m^2$ to 89±21 ml/$m^2$.[220] This improvement in volume status has the potential to alter cardiac loading conditions favourably. The reduction of preload and afterload are shown to cause cardiac reverse remodelling in heart failure.[223] Similar cardiac reverse remodelling after RTx has been proposed.[221]
- Correction of anaemia: Improved erythropoiesis after RTx corrects anaemia[224] and some of the cardiac benefits of RTx is attributable to anaemia correction.[221]

- Correction of oxidant stress: Heart failure research has shown that oxidative stress causes myocardial injury.[69,71] It has been proposed that oxidative stress is a potential mediator of cardiotoxicity in CKD as well.[75,225] There is evidence to suggest RTx reduces oxidative stress .[226,227] This reduction in oxidative stress may confer some cardiac benefits post RTx.
- Impact on sympathetic and RAAS activation: RTx is not shown to reduce sympathetic[228] or RAAS activation[229] hence it is less likely to play a role in the cardiac benefits of RTx.
- Uraemic toxins: There is growing interest in the role of uraemic toxins, especially protein bound uraemic toxins (PBUTs), in the evolution of cardiac disease in CKD. It is pertinent to note that RTx is the only renal replacement therapy modality that has been shown to remove PBUTs. The cardiotoxicity of uraemic toxins are discussed further in the subsequent chapters of the thesis.

In addition to showing cardiac functional improvement, the results also showed that improvement in cardiac function occurred in the absence of statistically significant alterations in peak $O_2$ consumption ($VO_{2max}$), revealing a discordance between $CPO_{max}$ and $VO_{2max}$. This reflects the fact that $VO_{2max}$ is an indirect, surrogate marker of cardiac function and factors other than cardiac function, such as skeletal muscle mass and performance, haemoglobin and peripheral $O_2$ extraction etc. act as important determinants of $VO_{2max}$.[230] The lack of improvement in $VO_{2max}$ in the present study may be related to non-cardiac factors such as lack of improvement in haemoglobin or muscle deconditioning. Hence, the failure of $VO_{2max}$ to show

improvement with RTx[231] does no longer mean that cardiac dysfunction has not improved with RTx.

## 4.5 Limitations

In view of the small sample size I was not able to evaluate in detail the determinants of cardiac functional improvement brought about by renal transplantation. The small sample size also precluded analysis of the effects of medications. Further large studies would be worthwhile in the future.

The sample size in the present study is small as I studied the participants from study 1 with no co-morbid cardiac disease or DM who underwent renal RTx during the study period. The centre performs >100 renal transplantations per year. In the future studies, sample size can be significantly increased by not excluding patients with cardiovascular comorbidities or by involving multiple centres.

The CKD patients did not have echocardiographic assessment post RTx. Therefore, the effect of RTx on cardiac structure was not evaluated in this cohort.

## 4.6 Conclusion

The results of study 2 of the thesis support the hypothesis that renal transplantation improves peak cardiac power and cardiac reserve. Further large studies are required in the future to evaluate in detail the factors that influence changes in cardiac structure, function and composition in CKD patients following RTx.

# CHAPTER 5

# Study 3

# Echocardiographic abnormalities of cardiac structure and function in asymptomatic chronic kidney disease patients and their association with peak cardiac performance

## 5.1 Introduction

As described in the introduction (Chapter 1) echocardiographic studies have shown a high prevalence of LVH in patients with CKD. The prevalence and the severity of LVH were shown to increase with increasing severity of CKD and helped establish LVH as the predominant structural characteristic of UCM.[117-119] These structural changes were seen even in asymptomatic patients. However, their corresponding functional correlate was not identifiable.[119] Therefore, it was not known whether the LVH was accompanied by normal or abnormal cardiac function. Hence, there remains an important unanswered question whether the cardiac remodelling in CKD is physiological or pathological.

Furthermore, most echocardiographic studies in CKD did not exclude patients with cardiovascular comorbidities and hence it is hitherto unknown whether the echocardiographic abnormalities demonstrated in previous studies were a consequence of cardiovascular comorbidities or CKD per se.

In study 3 of this thesis, I evaluated the cardiac structure and resting cardiac function of asymptomatic CKD patients without comorbid cardiac disease or diabetes using echocardiography. I then correlated these findings with measures of cardiac (dys)function obtained by assessments of peak cardiac performance as discussed in Study 1 of the thesis (Chapter 2).

## 5.2 Methodology

Study design: A cross sectional study of a subgroup of CKD patients (n=54) who underwent cardiopulmonary exercise testing (CPX) for Study 1.

Investigations

*CPX:* CPX were performed as discussed in the methodology section.

*Echocardiography:* Echocardiography was performed by a qualified senior echocardiographer who was blinded to the severity of CKD of the study participants. A 2-dimensional study was performed in the standard parasternal long axis and short axis planes followed by an apical 4-chamber, apical 5-chamber and apical 2-chamber planes to evaluate cardiac structure and left ventricular contractility. In the parasternal long axis M-mode studies were performed in the left ventricle, at the end of the mitral tips. A frozen M-mode image was used to measure ventricular wall thickness and chamber size, in end diastole (EDD) at the peak of the R wave on the ECG and end systole (ESD) at the point of closest approximation of the posterior wall and the interventricular septum. Left atrial size was measured at the widest point during atrial diastole. Left ventricular ejection fraction (LVEF) was calculated by Simpson's biplane method[185] and left ventricular mass index (LVMI) was calculated by Devereaux method.[186] The above data was utilised in evaluating the presence of the following 4 geometric patterns in CKD subjects. Normal LV geometry (normal LV mass and lower value of relative wall thickness), eccentric LV hypertrophy (increased LV mass and lower value of relative wall thickness), concentric LV hypertrophy (increased LV mass and increased relative wall thickness) and concentric LV remodelling (normal LV mass and increased relative wall thickness).[232]

Statistical analysis: Difference in echo parameters between study groups was evaluated using independent sample t-test. The correlation between echo and biochemical parameters was evaluated using Pearson's correlation. Results are presented as mean±SD. $P<0.05$ is considered as significant.

## 5.3 Results

The body composition and age of study participants are presented in Table 5.1. The BMI and BSA of the CKD groups were comparable. However, the CKD 5 patients were older than other 2 groups. There was clear distinction in CKD related biochemistry between groups with varying severity of CKD (Table 5.1). No difference in resting systolic, diastolic or mean blood pressure existed between the CKD study groups (Table 5.1).

**Table 5.1** Body composition and biochemistry of study participants

| | CKD 2-3a (n=17) | CKD 3b-4 (n=24) | CKD 5 (n=13) |
|---|---|---|---|
| Age (year) | 42.2±11.6 | 48.9±11.3 | 55.8±11.4 |
| BMI ($kg/m^2$) | 27.5±4.2 | 27.8±4.2 | 28.6±4.4 |
| BSA ($m^2$) | 2.06±0.16 | 2.00±0.19 | 2.06±0.12 |
| Systolic BP (mmHg) | 111.8±11.8 | 112.9±12.7 | 114.6±13.3 |
| Diastolic BP (mmHg) | 72.9±8.5 | 72.5±6.8 | 70.3±7.8 |
| Mean arterial pressure (mmHg) | 88.9±8.0 | 89.2±8.2 | 88.5±8.0 |
| **Biochemistry** | | | |
| eGFR (ml/min) | 65.3±12.6 | 27.8±8.8 | 12.8±1.9 |
| Creatinine (umol/l) | 115.3±17.7 | 247.8±71.3 | 469.2±78.4 |
| Urea (mmol/l) | 8.6±2.4 | 17.7±5.5 | 24.9±4.5 |
| Haemoglobin (g/l) | 14.9±1.3 | 13.2±1.5 | 12.2±1.0 |
| Calcium (mmol/l) | 2.35±0.08 | 2.35±0.11 | 2.34±0.13 |
| Inorganic Phosphate (mmol/l) | 1.07±0.17 | 1.21±0.15 | 1.43±0.28 |
| PTH (pmol/l) | 14.3±27.1 | 19.2±15.6 | 37.0±27.7 |
| Bicarbonate (mmol/l) | 27.3±2.9 | 23.6±3.4 | 22.3±2.4 |
| Urine PCR (mg/mmol) | 21.5±28.8 | 92.8±116.4 | 123.7±84.0 |

BMI: body mass index, BSA: body surface area, CKD: chronic kidney disease, PTH: parathyroid hormone, PCR: protein creatinine ratio.

Left ventricular mass index (LVMI) increased with increasing severity of CKD

There was an increasing trend in LVMI with increasing severity of CKD. LVMI in CKD 5 was significantly higher than that of CKD 2-3a (P=0.03) and the difference with CKD 3b-4 did not reach statistical significance for either of the groups either side (Table 5.2 & Fig 5.1). The proportion of patients with cardiac remodelling or left ventricular hypertrophy (LVMI >116 $g/m^2$) increased with increasing severity of CKD. Only 23.1% of CKD 5 patients had a structurally normal heart compared to 50% of CKD 3b-4 and 64.7% of CKD 2-3a (Fig 5.2).

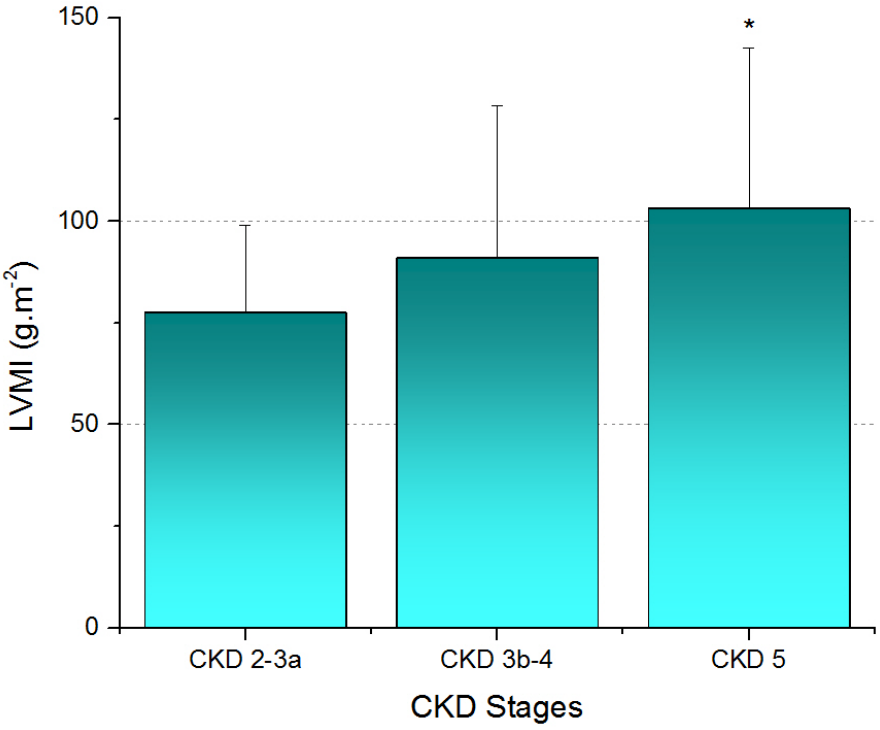


**Figure 5.1:** Left ventricular mass index (LVMI) increased with increasing severity of chronic kidney disease (CKD). *P<0.05 vs CKD 2-3a

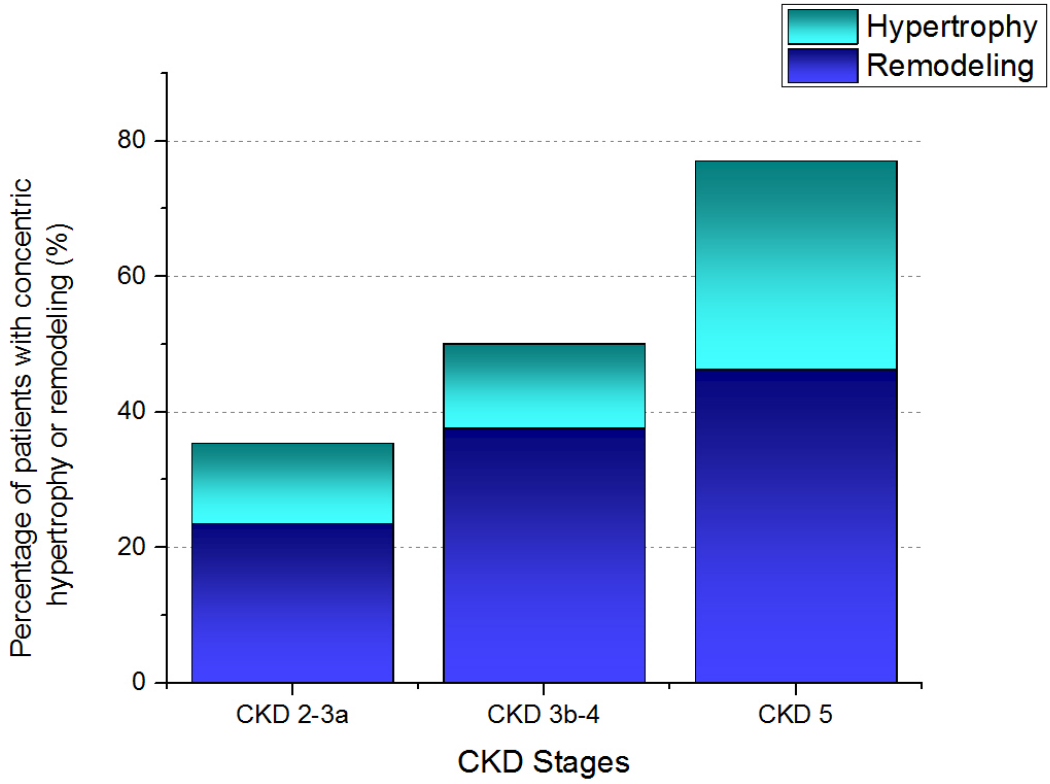


**Figure 5.2:** Proportion of patients with concentric remodelling or left ventricular hypertrophy increased with increasing severity of CKD.

**Table 5.2:** Cardiac structure and function of study participants

| | CKD 2-3a (n=17) | CKD 3b-4 (n=24) | CKD 5 (n=13) |
|---|---|---|---|
| **Structure** | | | |
| LVMI ($g/m^2$) | 77.5±21.6 | 90.9±37.6 | 103.1±39.4* |
| RWT (mm) | 4.24±1.12 | 4.25±0.94 | 4.77±1.15 |
| IVSD (mm) | 9.85±2.15 | 10.92±2.88 | 12.63±2.38* |
| LVEDD (mm) | 46.85±4.61 | 47.65±6.11 | 47.08±6.66 |
| LVSD (mm) | 33.92±4.35 | 32.88±4.84 | 30.93±7.74 |
| LA (mm) | 34.31±3.92 | 36.96±5.20 | 38.15±4.65* |
| **Resting function** | | | |
| LVFS (%) | 27.6±6.6 | 30.84±6.96 | 33.81±15.84 |
| Proportion with LVEF <50% (%) | Nil | Nil | Nil |
| E/A ratio | 1.12±028 | 1.08±0.37 | 0.84±0.32 |
| Proportion with E/A ratio <1 (%) | 17.6 | 45.8 | 69.2 |
| Resting CPO (W) | 1.10±0.28 | 0.92±0.14 | 0.92±0.22 |
| **Peak function** | | | |
| Peak cardiac output (l/min) | 21.3±2.3 | 19.7±1.9 | 18.3±2.1 |
| $CPO_{max}$ (W) | 4.94±0.77 | 4.61±0.47 | 4.05±0.68* |
| $\%CPO_{max}$ (%) | 93.9±12.7 | 88.6±10.0 | 80.4±13.9* |

LVMI: left ventricular mass index, RWT: relative wall thickness, IVSD: interventricular septal dimension, LVEDD: left ventricular end diastolic diameter, LVSD: left ventricular systolic diameter, LA: left atrial diameter, LVFS: left ventricular fractional shortening, LVEF: left ventricular ejection fraction, E/A ratio: ration of early (E) to late (A) ventricular filling velocities, CPO: cardiac power output. *P<0.05 vs CKD 2-3a on Independent sample t-test.

Other measures of cardiac dimension amongst study groups

Interventricular septal thickness and left atrial diameter was higher in CKD 5 compared to CKD 2-3a ($P<0.05$). The difference between CKD 5 and CKD 3b-4 did not reach statistical significance. The relative wall thickness (RWT), left ventricular end diastolic diameter and left ventricular systolic diameter were comparable between study groups (Table 5.2).

Peak cardiac power output ($CPO_{max}$) decreased with increasing severity of CKD

$CPO_{max}$ in CKD 5 was significantly impaired compared to CKD 2-3a ($P=0.003$) and CKD 3b-4 ($P=0.006$). Figure 5.3 shows the concomitant increase in LVMI and decrease in $CPO_{max}$ with increasing severity of CKD.

Association between LVMI and haemodynamic parameters

There was no statistically significant correlation between LVMI and the haemodynamic parameters such as $CPO_{max}$ ($r=-0.115$, $P=0.12$), peak CO ($r=-0.186$, $P=0.18$), peak HR ($r=-0.172$, $P=0.21$) or peak MAP ($r=0.08$, $P=0.5$).

Cardiac power-to-mass ratio in CKD

Peak cardiac power per 100 gram of LVM showed significant positive correlation with eGFR ($r=0.44$, $P=0.001$) (Fig 5.4). Peak cardiac power per 100 gram of LVM also showed graded decline across CKD stages with 3.28±0.9 W/100g in CKD 2-3a, 2.83±0.9 W/100g in CKD 3b-4 and 2.13±0.8 W/100g in CKD 5 (Fig 5.5). It also showed significant negative correlation with urea ($r=-0.48$, $P<10^{-3}$) and inorganic phosphate ($r=-0.42$, $P=0.002$), and positive correlation with bicarbonate ($r=0.29$, $P=0.03$). No significant correlation was seen with haemoglobin, serum calcium and urine protein creatinine ratio.

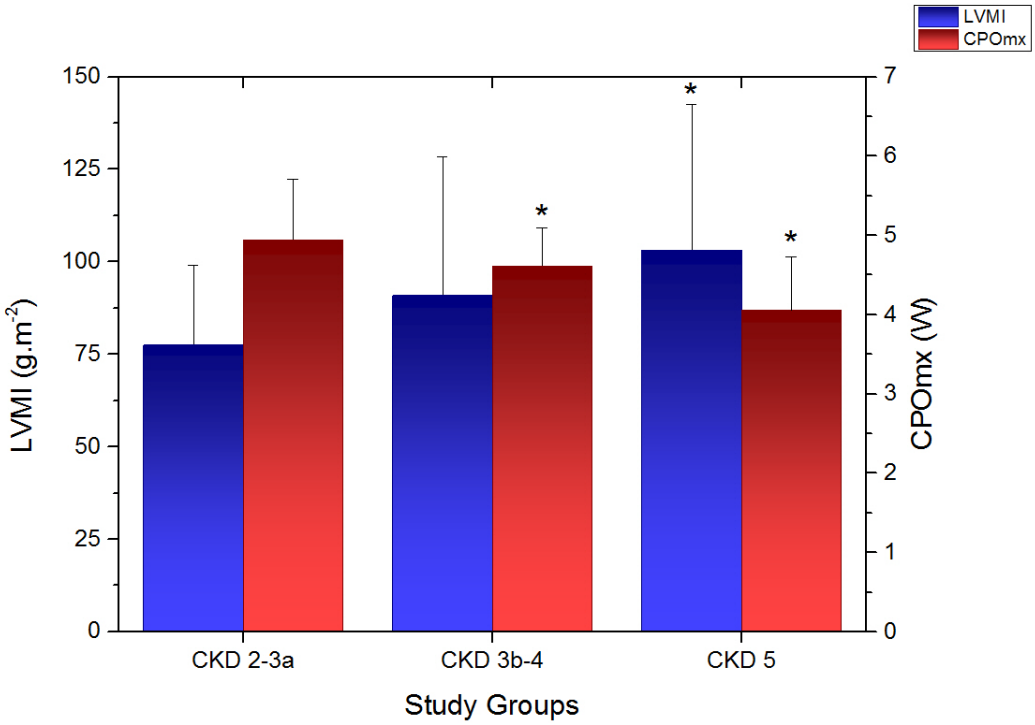


**Figure 5.3:** Increase in left ventricular mass index (LVMI) and decrease in peak cardiac power output ($CPO_{max}$) across the study groups. *P<0.05 vs CKD 2-3a on Independent sample t-test.

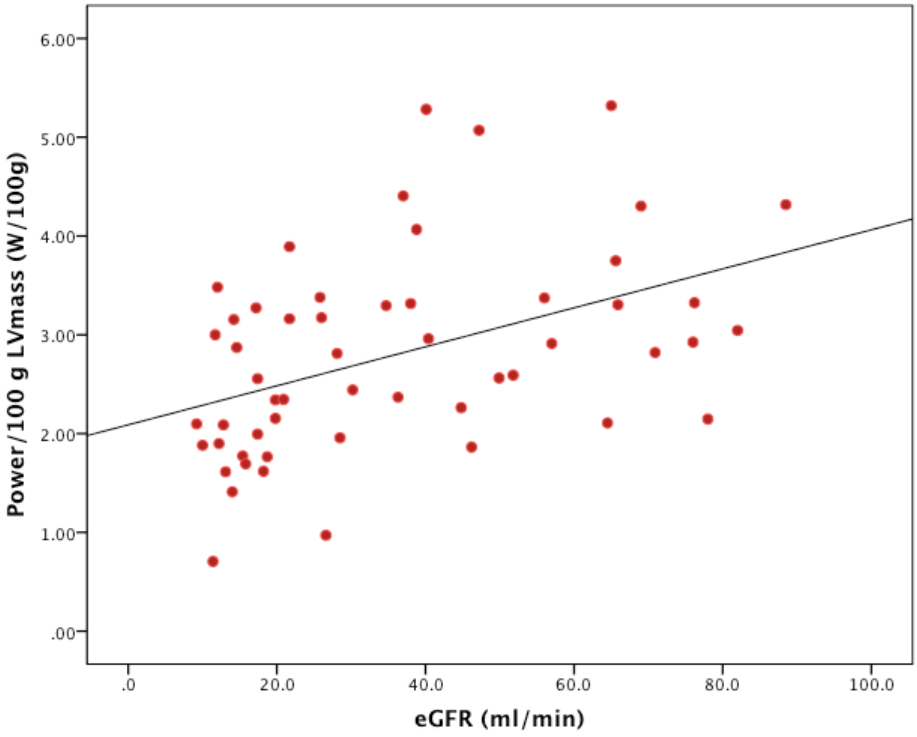


**Figure 5.4:** Association between eGFR and power/100 g of LVM. R=0.44, P=0.001 on Pearson's correlation.

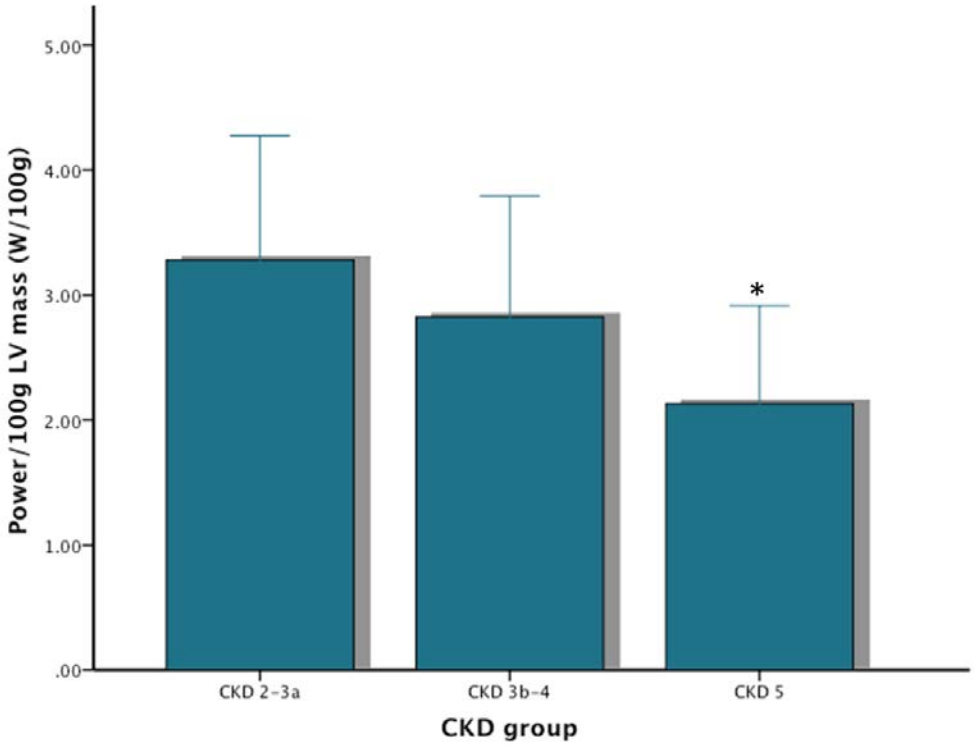


**Figure 5.1:** Cardiac power per 100 g of LVM decreased with increasing severity of chronic kidney disease (CKD). *P<0.05 vs CKD 2-3a on Independent sample t-test.

Association between LVMI and CKD-related biochemistry

LVMI had significant negative correlation with eGFR and serum calcium, and significant positive correlation with urea and inorganic phosphate. There was no correlation with haemoglobin, parathyroid hormone, calcium and urine protein creatinine ratio (Fig 5.4).

Association between left ventricular mass and demographic parameters

Left ventricular mass correlated with BMI (r=0.31, P=0.02) and BSA (0.33, P=0.02). There was no correlation between LVM or LVMI with age. Relative wall thickness (RWT) correlated with age (0.37, P=0.007). There was no correlation between LVM or LVMI with systolic, diastolic and mean blood pressure. RWT correlated with mean blood pressure (r=0.31, P=0.03).

Association between LVMI and antihypertensive medications

There was no significant difference in LVMI between patients who were on ACE-i (n=32) compared to patients not on an ACE-i (n=22). The LVMI were 89.6±37.4 vs 89.7±37.1 $g.m^{-2}$ (P=NS). Similarly, there was no significant difference in LVMI between patients who were on an ARB (n=21) compared to patients not on ARB (n=33). The LVMI were 91.6±31.8 vs 88.4±36.7 $g.m^{-2}$ (P=NS). The groups had comparable eGFR. There were 11 patients who were on beta blockers but the beta blocker group had significantly lower eGFR 21.4 ±15.5 ml/min compared to patients not on beta blockers 39.7±22.9 ml/min. Although the LVMI of beta blocker group was 22.6 $g. m^{-2}$ higher compared to the non beta blocker group, controlling for eGFR, the difference did not reach statistical significance (P=0.058).

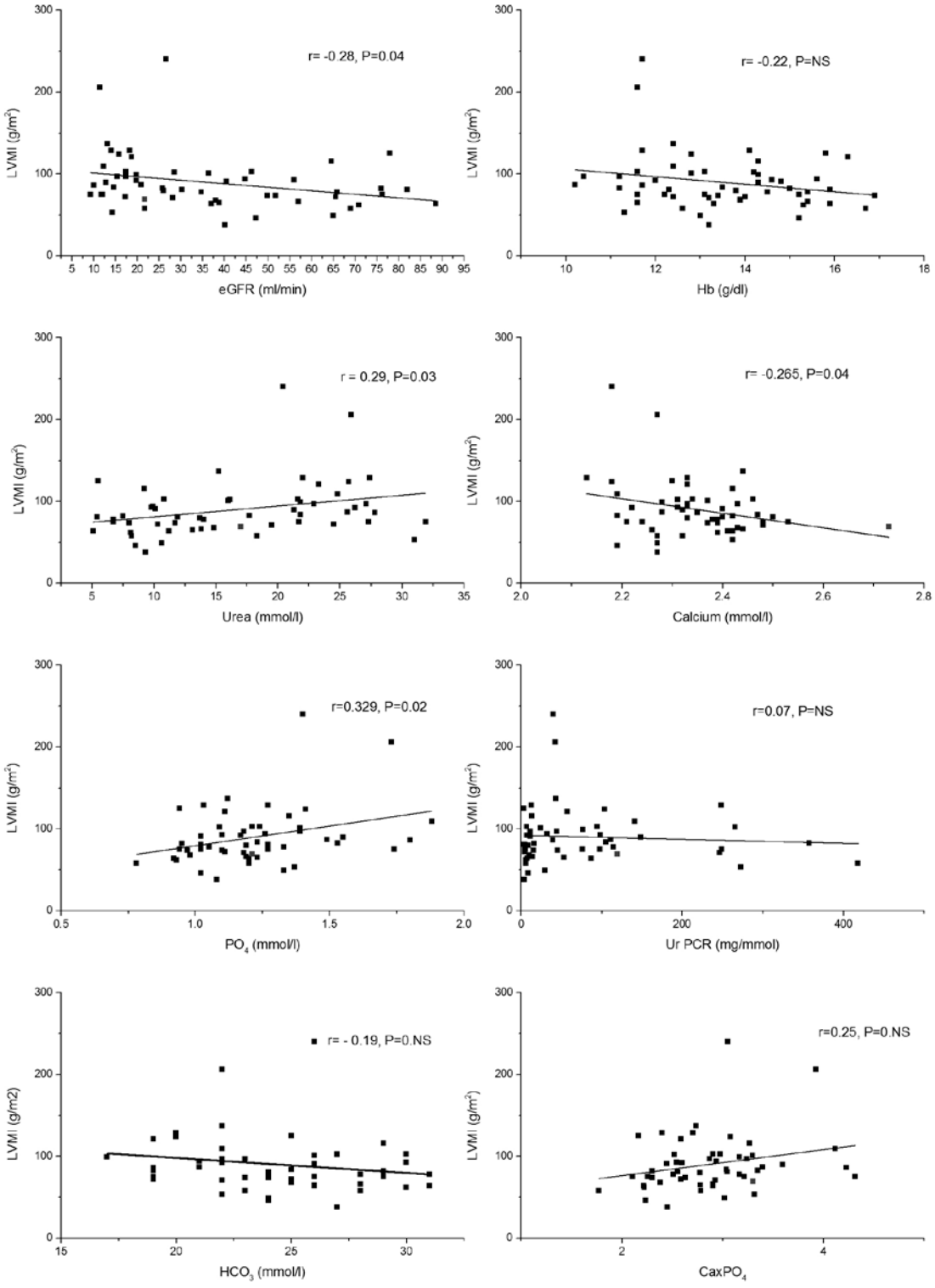


**Figure 5.6:** Association between left ventricular mass index (LVMI) and CKD-related biochemical parameters. Hb: haemoglobin, Ur PCR: urine protein creatinine ratio.

## 5.4 Discussion

The study supports our hypothesis that cardiac remodelling and hypertrophy occur in asymptomatic CKD even in the absence of co-morbid cardiac disease or diabetes. The prevalence of LVH and the LVMI increased with increasing severity of CKD. All of these asymptomatic patients had normal resting systolic cardiac function as evidenced by LVEF >50%. The LVMI appears to be negatively correlated with eGFR and positively correlated with urea and inorganic phosphate. No correlation existed with haemoglobin, urine protein creatinine ratio, serum bicarbonate, calcium, and calcium and phosphate product.

As discussed in Chapter 1 (section 1.5.2), an important characteristic that differentiates physiological/adaptive hypertrophy from pathological/maladaptive hypertrophy is the cardiac function. Whereas adaptive hypertrophy results in normal or supra normal function, maladaptive hypertrophy results in impaired cardiac function. The results of the present study demonstrate that the cardiac power-to-mass ratio is positively correlated with the eGFR. There is also graded decline across the CKD stages (Fig 5.5). Although the left ventricle appears to be hypertrophied in CKD, power per gram of left ventricular myocardium is significantly impaired especially in advanced CKD thus demonstrating the presence of pathological or maladaptive LVH.

Advances in heart failure research has revealed several possible mediators of pathological cardiac hypertrophy[118] and it is pertinent that most of these mediators are active in CKD as well, the most important stimulus being cardiac wall stress.[205] CKD causes renin angiotensin aldosterone system activation (RAAS), and sodium and water retention that leads to increased cardiac preload and afterload, in other words, volume and pressure overload respectively. The adaptation to the former is

eccentric hypertrophy aimed at increasing stroke volume and the latter is concentric hypertrophy aimed at minimizing the wall stress. The initial adaptive changes aimed at optimizing cardiac performance later become maladaptive leading to myocardial dysfunction. The precise mechanisms responsible for the transition from adaptive hypertrophy to maladaptive heart failure are not known, but there are several candidate mechanisms. Deficiencies in high-energy phosphate stores, defects in excitation-contraction coupling and excess formation of myocyte microtubules, which impairs sarcomere shortening are some of the proposed mechanisms.[233-235] Evidence exists demonstrating impaired cardiac energetics[137] and defective excitation-contraction coupling in uraemia.[140]

In addition, there are several neurohumoral mediators that are active in CKD with the potential to cause pathological hypertrophy. These include noradrenalin, angiotensin, endothelin, fibroblast growth factor and proinflammatory cytokines.[84,201,203] These mediators, in heart failure, appear to act through various signal transduction proteins to activate a family of enzymes that induce the 'fetal gene program'.[236] Fetal gene program represents an induction of a 'fetal' pattern of gene expression, whereby certain contractile, calcium-handling, and counter-regulatory proteins revert to the mRNA and protein expression pattern that characterizes the fetal stage of development. The net result of these changes is reduced myofilament ATPase activity and contractile velocity leading to myocardial dysfunction.[237] The myocardial dysfunction in turn leads to reduced cardiac output and further stimulation of the neurohumoral system setting up a vicious cycle (Fig 5.7) causing progressive failure of the myocardium.[118]

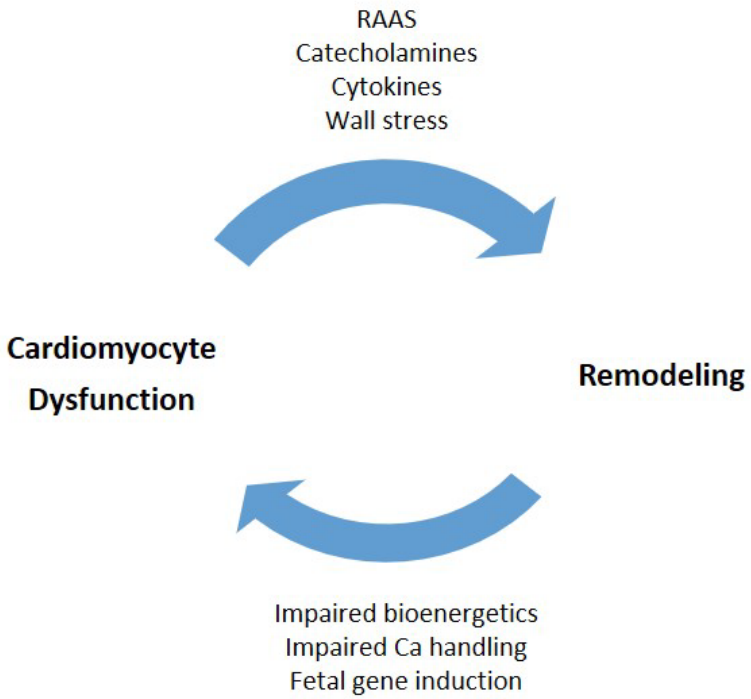


**Figure 5.7:** Vicious cycle of pathological hypertrophy and myocardial dysfunction.[118]

Drugs that block the neurohumoral activation such as beta receptor blockers and ACE inhibitors, that are shown to reverse this pathological remodelling, are the cornerstone of heart failure management.[210-212] However, the evidence for such treatment in uraemic cardiomyopathy is still lacking.

## 5.5 Limitations

As the study is cross sectional in nature it did not allow detection of transition from adaptive to maladaptive hypertrophy and the factors affecting it. A longitudinal study evaluating such transition would be worthwhile in the future. Furthermore, only routine echocardiographic assessment was performed on the study subjects and hence detailed description of diastolic dysfunction is not available. Techniques such as myocardial speckle tracking or tissue doppler imaging could offer further information in future studies.

I acknowledge that echocardiographic measures of cardiac dimensions are subject to inter observer variability. The echocardiographic assessment in the present study was performed by a senior echocardiographer who was blinded to the severity of CKD of the study subjects that helped in minimising bias. Further evaluation using cardiac MRI would be worthwhile in the future to obtain more accurate information on cardiac size as well as cardiac composition.

Although the use of anti hypertensive medication is not found to be a confounder in the association between LVMI and renal function in the present study, further longitudinal studies are required to evaluate the long-term effects of medications such as ACE-i, ARB or beta blocker on LVMI in CKD.

## 5.6 Conclusion

The study demonstrated the presence of cardiac remodelling and hypertrophy in asymptomatic CKD patients even in the absence of cardiovascular comorbidities. The study also showed concomitant cardiac dysfunction suggesting a pathological hypertrophy. Further studies are needed to explore the mechanisms of pathological hypertrophy in CKD, and the benefits of existing heart failure therapies and the development of novel interventions.

# CHAPTER 6

# Study 4

# Association between cardiac dysfunction and protein bound uraemic toxins in asymptomatic chronic kidney disease patients

## 6.1 Introduction

Cardiovascular disease (CVD) is the predominant cause of mortality and morbidity in chronic kidney disease (CKD) and end stage renal disease (ESRD). Several pathophysiological mechanisms are being studied to understand the mechanism of CVD in CKD. Among the potentially putative agents of CVD, there is an emerging body of evidence demonstrating the role of elevated levels of protein bound uraemic toxins (PBUTs) such as indoxylsulphate (IXS), p-cresylsulphate (PCS), p-cresyl glucuronide (PCG), hippuric acid, indole acetic acid, CMPF etc.[225,238-241]

There are over 30 PBUTs identified so far with their protein binding varying from 10% (e.g., PCG) to >9O% (e.g., IXS). It is the free (unbound) fraction that is biologically active and elicits toxicity. Of the PBUTs, indoxyl sulphate (IXS) and p-cresyl sulphate (PCS) are the most studied toxins.[91] They are shown to elicit a wide range of toxicity such as endothelial dysfunction, vascular calcification and induction of oxidative stress.[93-95 96,97] More importantly, clinical studies have shown that IXS and PCS are associated with cardiovascular and all-cause mortality.[95,98] Furthermore, serum IXS levels were shown to be associated with incidence of heart failure in CKD patients.[242] However, the association between cardiac dysfunction and PBUTs has not yet been studied.

In Study 4 of this thesis, I evaluated the association between impaired peak cardiac function and the serum free and total concentrations of potentially cardiotoxic PBUTs.

## 6.2 Methodology

Full methodology is presented in chapter 2 of the thesis. A brief methodology relevant to this chapter is presented here. In a subset of 56 CKD patients (stages 2-5), who participated in study 1 of the thesis, we measured both $CPO_{max}$ and serum levels of protein-bound uraemic toxins to evaluate the association between them.

Cardio-pulmonary exercise tests (CPX)

Peak cardiac power was measured using non-invasive maximal cardiopulmonary exercise testing as described in Chapter 2.

Protein-bound uraemic toxin assays

Sample collection: blood samples were obtained from study subjects prior to CPX exercise testing. The samples were immediately centrifuged and the serum stored as 0.5 ml aliquots in a -80°C freezer. Indoxylsulphate (IXS), p-cresyl sulphate, p-cresyl glucuronide, indole acetic acid, hippuric acid, uric acid and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF) were assayed in Ghent University Hospital, Ghent, Belgium as described in the previously published methodological papers [90,243].

HPLC Analysis: chromatographic analyses were performed on the above stored serum samples. For quantification of the total fraction, serum samples were deproteinised by heat (95°C, 30 min). After cooling (10 min on ice), samples were filtered through a Centrifree-filter (cut-off: 30 kDa; Millipore, Billerica, MA). The ultrafiltrate was injected onto the column. To determine the free fraction, untreated serum samples were ultrafiltered prior to heating. Indoxyl sulphate, indole-3-acetic acid, p-cresyl sulphate and p-cresyl glucuronide were detected by fluorescence, while hippuric acid, CMPF and uric acid were detected by UV [Waters Alliance 2695

device (Waters, Zellik, Belgium) connected to a Waters fluorescence and a UV detector].

### 6.2.1 Statistical analysis

Clinical study: $CPO_{max}$ was the primary outcome measure. Statistical analysis was performed as described in Chapter 2 (page 80).

## 6.3 Results

Patient characteristics

There were 56 male CKD patients with a mean age of 46.8±12.5 years covering the spectrum of CKD from stages 2 to 5 (CKD 2-5, pre-dialysis). The mean eGFR was 38.5±24.1 ml/min. The eGFR, and the free and total (protein bound) concentrations of the uraemic toxins are presented in Table 6.1.

Association between $CPO_{max}$ and uraemic toxins

The correlation between $CPO_{max}$ and total and free concentrations of uraemic toxins are shown in Figures 6.2 and 6.3 respectively. Indoxyl sulphate (IXS) and p-cresyl sulphate showed strong negative correlation with $CPO_{max}$ (Fig 6.2 & 6.3). No correlation existed between $CPO_{max}$ and total or free concentrations of uraemic toxins such as p-cresyl glucuronide, indole acetic acid, hippuric acid, uric acid or 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF). A multivariate analysis [using the partial least squares (PLS) multiple regression method] was performed and the following uraemic toxins were found to be associated with $CPO_{max}$ independent of eGFR, all $P<0.05$ ($\beta$, standardized co-efficient in brackets): free indoxyl sulphate (IXS) (-0.25), free p-cresyl sulphate (PCS) (-0.32), total IXS (-0.24) and total PCS (-0.36).

**Table 6.1** Total and free concentrations of the assayed protein-bound uraemic toxins across CKD groups (n=56).

| | | CKD 2-3a (n=20) | CKD 3b-4 (n=25) | CKD 5 (n=11) |
|---|---|---|---|---|
| eGFR (ml/min) | | 66.9±12.5 | 27.4±8.9 | 11.9±2.4 |
| Uraemic toxins | | | | |
| IXS (mg/dl) | Total | 0.10±0.05 | 0.37±0.21 | 1.44±1.13 |
| | Free | 0.002±0.001 | 0.009±0.005 | 0.05±0.05 |
| PCS (mg/dl) | Total | 0.35±0.23 | 1.21±0.9 | 1.67±1.04 |
| | Free | 0.009±0.006 | 0.029±0.023 | 0.043±0.031 |
| PCG (mg/dl) | Total | 0.01±0.01 | 0.024±0.02 | 0.09±0.1 |
| | Free | 0.004±0.004 | 0.019±0.016 | 0.077±0.114 |
| IAA (mg/dl) | Total | 0.04±0.01 | 0.10±0.06 | 0.12±0.12 |
| | Free | 0.003±0.001 | 0.011±0.009 | 0.018±0.018 |
| Hippuric acid (mg/dl) | Total | 0.14±0.09 | 0.32±0.16 | 0.48±0.29 |
| | Free | 0.046±0.035 | 0.117±0.068 | 0.166±0.114 |
| CMPF (mg/dl) | Total | 0.10±0.10 | 0.11±0.11 | 0.11±0.08 |
| Uric Acid (mg/dl) | Total | 9.65±2.43 | 9.86±2.29 | 10.36±2.19 |

Indoxylsulphate (IXS), p-cresyl sulphate (PCS), p-cresyl glucuronide (PCG), indole acetic acid (IAA), hippuric acid, uric acid and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF)

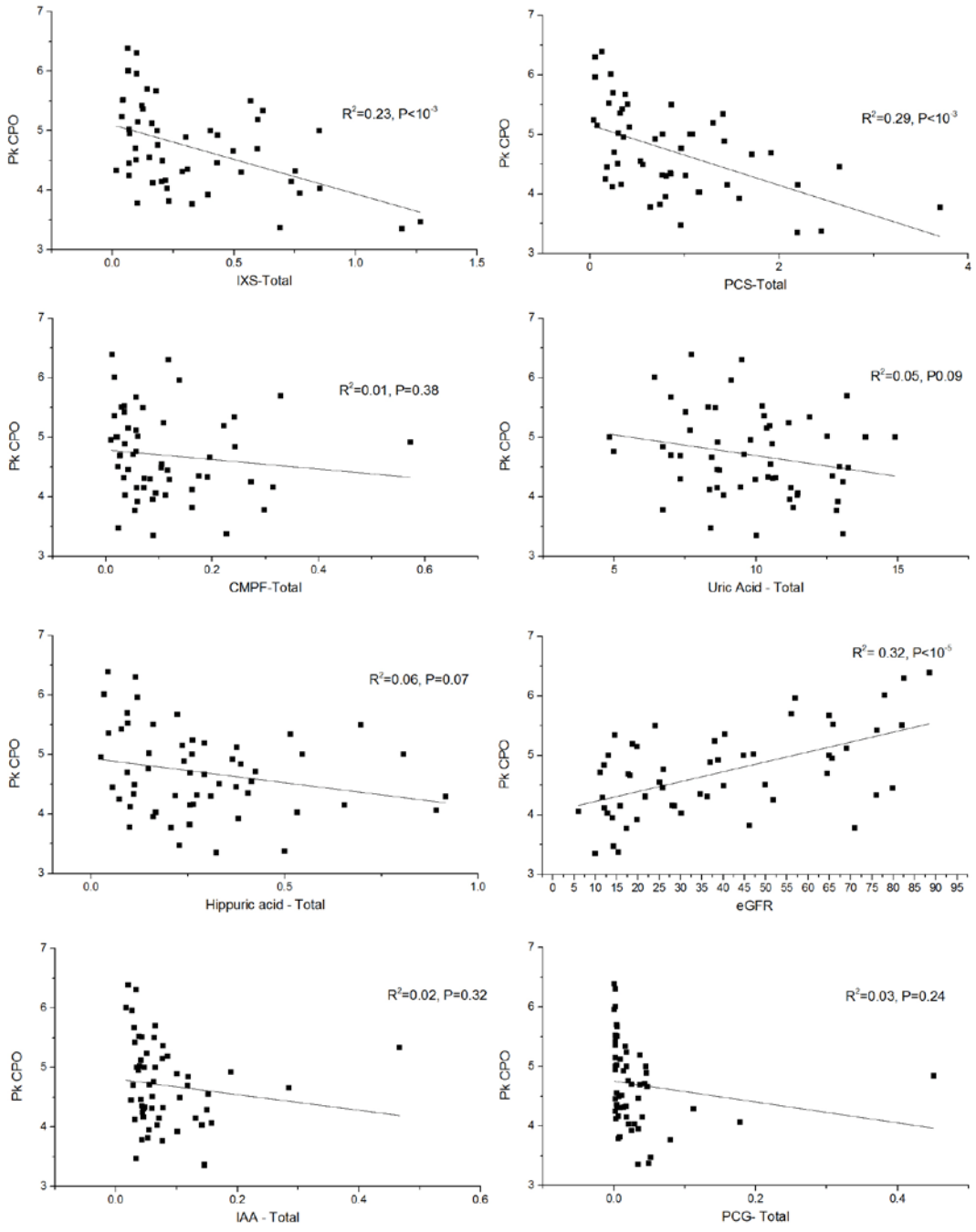


**Figure 6.1:** Association between $CPO_{max}$ and total concentrations of uraemic toxins. Indoxylsulphate (IXS), p-cresyl sulphate (PCS), p-cresyl glucuronide (PCG), indole acetic acid (IAA), hippuric acid, uric acid and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF)

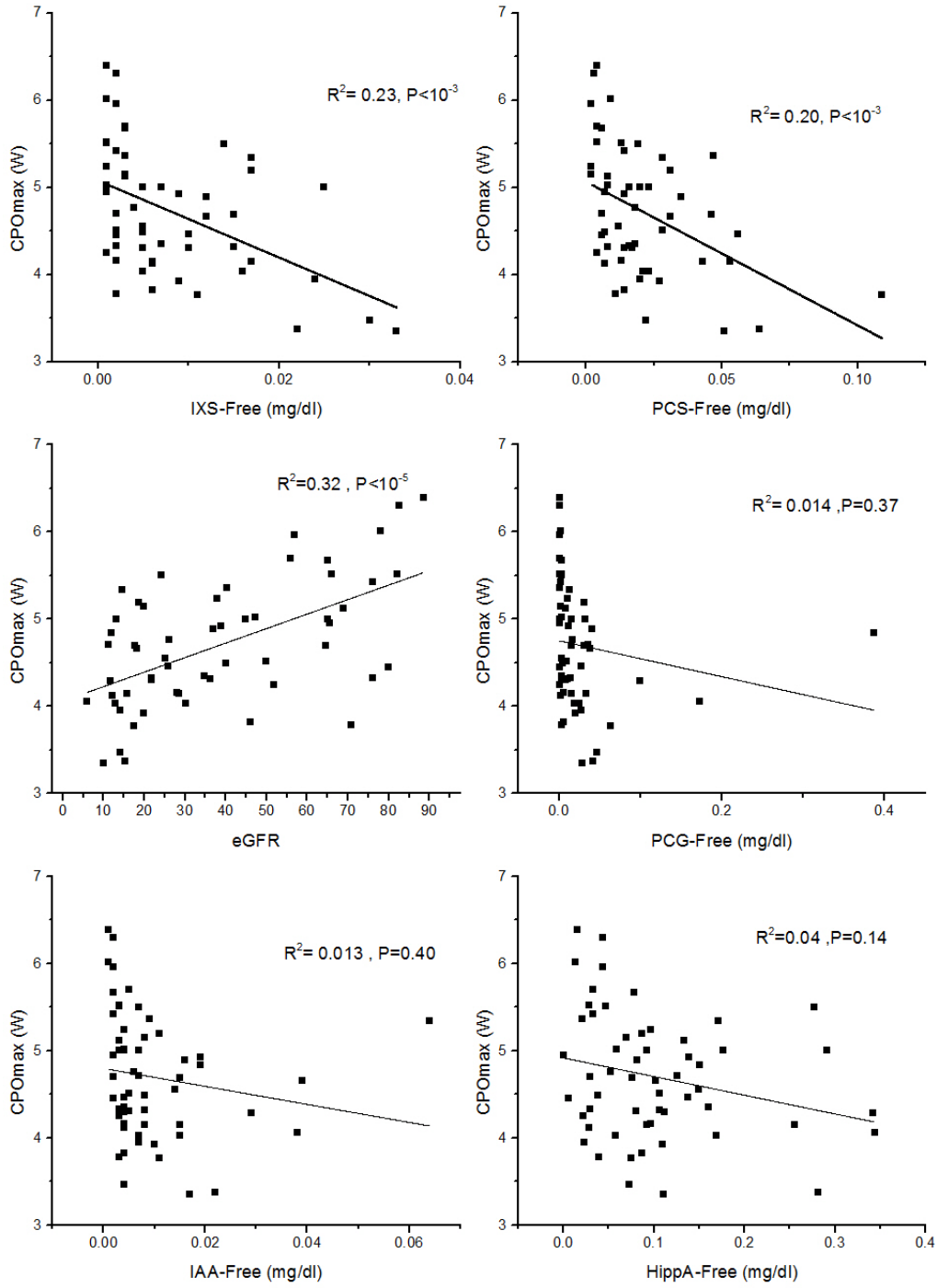


**Figure 6.2:** Association between $CPO_{max}$ and free concentrations of uraemic toxins. Indoxylsulphate (IXS), p-cresyl sulphate (PCS), p-cresyl glucuronide (PCG), indole acetic acid (IAA), hippuric acid, uric acid and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF)

**Association between protein bound uraemic toxins and LVMI**

A sub group of 49 patients had echocardiographic assessment in addition to CPX. There was no correlation between serum levels of protein bound uraemic toxins and LVMI in the study cohort.

## 6.4 Discussion

The results demonstrate the independent association between protein bound uraemic toxins and cardiac dysfunction in CKD. Of the 30 or more protein-bound uraemic toxins that have previously been reported,[244] we tested the leading candidates that might be cardiotoxic, and we found both free and total IXS and PCS were independently and significantly associated with subclinical cardiac dysfunction in CKD.

Although it has been shown in the past that IXS is associated with incidence of HF in CKD,[242] no association between IXS levels and LVEF or serum B type natriuretic peptides (BNP) were shown.[245] The present study, for the first time has shown the association between serum IXS and PCS levels and sub clinical cardiac dysfunction in CKD.

With regards to cardiac structure, a study in the past had demonstrated reduction in the prevalence of LVH in CKD patients treated with gut uraemic toxins binder AST-120.[134] However, uremic toxin levels were not measured in the study. The association between serum uraemic toxin levels and LVH in CKD patients was hitherto unknown. The present study showed that there is no correlation between uraemic toxins and LVMI in asymptomatic CKD patients.

IXS and PCS are generated in the gut by the action of intestinal flora on the substrates tryptophan and tyrosine respectively.[246] These toxins are then absorbed and bind avidly with serum albumin. On an average 90% of these toxins are protein

bound. The remaining 10% unbound free toxin is biologically active.[247] The normal mechanism of removal of these toxins by the kidneys is still being evaluated. It is suspected that these toxins are predominantly removed by tubular secretion with organic anion transporters (OAPs) playing an important role.[248]

There are several potential mechanisms by which these uraemic toxins could elicit cardiotoxicity and some of these mechanisms have already been elucidated. In isolated cardiac tissue, IXS caused fibrosis and hypertrophy in neonatal rat heart[99] where inhibition of AMPK signalling was implicated.[100] In adult rat hearts, fibrosis and hypertrophy were linked to reactive oxygen species (ROS) production and NFκB signaling.[101] Reducing IXS levels by AST-120 (oral charcoal) reduced NFκB phosphorylation and fibrosis in 5/6 nephrectomy rats.[102] Although the above studies demonstrated the role of PBUTs in inducing cardiac ultrastructural changes, studies on the effects of these toxins on the cardiomyocytes function are still lacking.

As PBUTs are mostly albumin bound, they are not as easily dialysed as small molecules or the middle molecules.[92] Due to the protein bound nature of these toxins, the free serum concentration bounces back soon after dialysis. Hence these toxins persist in the patients' sera and continue to elicit toxicity.[92] Therefore, further research is needed in finding ways of reducing the uraemic toxin concentration. Reducing the generation of these toxins by altering diet and by adding probiotics to diet has been proposed.[249] Intestinal adsorbents of these toxins such as AST 120 have been shown to reduce the levels of these toxins.[249] In addition, special dialysis techniques designed to remove the albumin bound fraction of PBUTs have also been proposed.[250] A prospective study of the effect of such toxin reduction strategies on cardiac function may be worthwhile in the future.

## 6.5 Limitations

The cross-sectional nature of the study only allows demonstration of association between uraemic toxins and cardiac dysfunction. Further longitudinal studies of uraemic toxin reducing strategies are required to demonstrate causal relationship between uraemic toxin and cardiac dysfunction.

## 6.6 Conclusion

The study demonstrates the association between elevated levels of PBUTs, indoxyl sulphate and p-cresyl sulphate and cardiac dysfunction in asymptomatic CKD patients. In the next chapter I describe the effect of indoxyl sulphate on the mechanical properties of isolated rat cardiomyocytes.

# CHAPTER 7
# Study 5

# Acute effects of the uraemic toxin, Indoxyl Sulphate, on the mechanical properties of isolated rodent cardiomyocytes

## 7.1 Introduction

Heart failure (HF) is the predominant cardiac disease of CKD with >50% prevalence in end stage renal disease (ESRD).[251] Dialysis does little to stop the relentless progression of heart disease in CKD.[9] The cardiorenal interaction is mediated by multiple factors and there is growing interest in the role of uraemic retention solutes, especially protein bound uraemic toxins (PBUTs),[90] as mediators of cardiotoxicity.[91] PBUTs are mostly bound to albumin and are not well cleared by dialysis and therefore build up in patients[92] and continue to elicit toxicity in spite of dialysis.

There is an emerging body of evidence emphasising the role of protein bound uraemic toxins such as indoxylsulphate (IXS), p-cresylsulphate, p-cresyl glucuronide, indole acetic acid, hippuric acid, etc in cardiovascular disease of CKD.[225,238-241] The deleterious effects of some of these toxins have been well studied.[91] The vasculotoxicity of IXS, an indole, is demonstrated by its ability to cause endothelial dysfunction, vascular calcification and induction of oxidative stress.[93-95]. Clinical studies have shown that IXS is associated with vascular calcification and vascular stiffening. The uraemic toxin has also been shown to predict cardiovascular and all-cause mortality.[95,98] Furthermore, IXS has been shown to be associated with cardiac dysfunction[252] in CKD and the incidence of de-novo heart failure in end stage renal disease (ESRD).[253] In addition, reducing circulating levels of IXS is shown to prevent the progression of left ventricular hypertrophy in patients with CKD. [134]

In animal studies, IXS has been shown to cause fibrosis and hypertrophy in isolated neonatal rat heart.[99,100] In adult rat hearts, fibrosis and hypertrophy were linked to reactive $O_2$ species (ROS) production and NFκB signalling.[101] AST-120 (oral charcoal) reduced IXS, NF-κB phosphorylation and fibrosis in 5/6 nephrectomy rats.[102] Although the above studies demonstrated the role of PBUTs in inducing cardiac ultrastructural changes, studies on the effects of these toxins and the associated mechanisms on the mechanical properties (i.e. the contraction) of cardiomyocytes are still lacking.

In study 4 of this thesis I demonstrated the association between subclinical cardiac dysfunction and serum levels of IXS and PCS. Although both these toxins showed independent association with cardiac dysfunction, as discussed above, there is a significant body of evidence highlighting the cardiotoxicity of IXS compared to PCS. Furthermore, IXS was shown to be associated with incident HF in CKD[242] making it a suitable candidate for further *in vitro* evaluation. Therefore, in this chapter of the thesis I tested the hypothesis that IXS has direct inhibitory effect on the mechanical properties of isolated rat cardiomyocytes.

## 7.2 Methodology

Detailed methodology is presented in Chapter 2 of the thesis. A concise methodology is presented here.

### 7.2.1 Cardiac myocytes collection

Single ventricular cardiac myocytes were isolated from male Wistar rats (approximately 250 g). Briefly, hearts were removed following sacrifice by Directive 2010/63/EU approved methods then Langendorff-perfused with a collagenase- and protease-containing solution. The ventricles and atria were then separated, and single

ventricular myocytes harvested. Myocytes were stored at 20–23 °C in storage solution (see below) and used within 10 h.

### 7.2.2 Experimental solutions

Myocytes were stored in a solution containing (in mmol/l): 130 NaCl, 5.4 KCl, 0.4 $NaH_2PO_4$, 1.4 $MgCl_2 \cdot 6H_2O$, 5 HEPES, 10 glucose, 10 taurine, 20 creatine and 0.7 $CaCl_2$ (pH adjusted to 7.3 with NaOH). The experimental (Tyrode) solution contained (in mmol/l): 137 NaCl, 5.4 KCl, 0.33 $NaH_2PO_4$, 0.5 $MgCl_2 \cdot 6H_2O$, 5 HEPES, 5.6 glucose, and 1.8 $CaCl_2$ (pH adjusted to 7.4 with NaOH). Indoxyl sulphate (IXS) (Sigma-Aldrich, Bornem, Belgium) was dissolved in saline to prepare the (300x) stock solution. IXS stock solution was tested for lipopolysaccharide contamination by a kinetic chromogenic Limulus ameobyte lysate test (Lonza, Vervier, Belgium). The effect of indoxylsulphate (IXS) was assessed by switching the superfusion solution to Tyrode solution containing IXS at a final concentration of 4.49 mg/l, the maximum reported *free* concentration in uraemia.[188-190]

### 7.2.3 Measurement of myocyte contractility

Cells were placed in a chamber on the stage of an inverted microscope (Eclipse TE300, Nikon, Japan) fitted with a 40x objective. The cell chamber was perfused with solutions from reservoirs and warmed by a heater prior to entering the chamber. All experiments with single myocytes were performed at 35-37 ºC.

Cells were considered viable for experimentation if they appeared rod shaped with clear striations and were quiescent in the absence of stimulation. A video image of the cells was acquired using a camera (MyoCam-S, IonOptIx, USA) mounted to the side port of the microscope and displayed on a computer monitor. Sarcomere length (SL) was measured by fast Fourier transform of an area of the cell, giving the

dominant frequency of oscillations in contrast between light and dark bands. This was automatically converted to a length in microns using IonWizard software (IonOptIx, USA). The cells were field stimulated via platinum bath electrodes using a 5 ms pulse and at a stimulation frequency 1 Hz. Diastolic SL, systolic SL, fractional shortening (defined as a percentage change in diastolic SL) and the time constant of an exponential fit to the time course of the relaxation (Tau) were measured by averaging 10 – 15 contractions in each cell.

### 7.2.4 Inhibition of Protein Kinase A

To inhibit the action of protein kinase A (PKA), cells were incubated in storage solution with 100 µmol/l Rp8-Br-cAMPS (Rp8, Santa Cruz Biotech, USA), for at least 30 minutes. The efficacy of Rp8 in inhibiting PKA activity in our experimental setting was verified by assessing its ability in blunting the positive inotropic effect of isoproterenol (Iso, 10 nmol/1), a non-specific β-adrenoceptor agonist.

### 7.2.5 Statistical analysis

Data are expressed as mean ± SEM. Statistical significance was tested using repeated measures ANOVA and independent sample t-tests where appropriate using SPSS statistics software version 17 (IBM, USA). Statistically significant differences were assumed when $P < 0.05$. Myocyte numbers for each experiment are given in the relevant figures and tables.

## 7.3 Results

### 7.3.1 Effect of IXS on the contraction of ventricular myocytes

#### Representative traces of cardiomyocyte contractions

Fig 7.1 shows a representative recording of the change in sarcomere length (SL) during exposure to and after wash out of IXS allowing the envelope of diastolic and systolic SL changes to be seen. Representative single contractions are shown in Fig. 7.2 & 7.3.

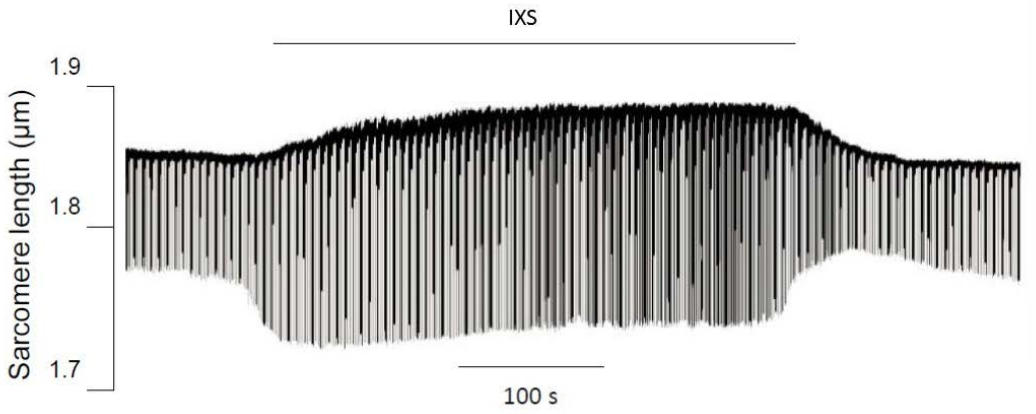


Figure 7.1: Representative example of the effect of exposure to and wash out of IXS on the sarcomere length (SL) of a single rat ventricular myocyte. Stimulation frequency 1 Hz, 37 ºC

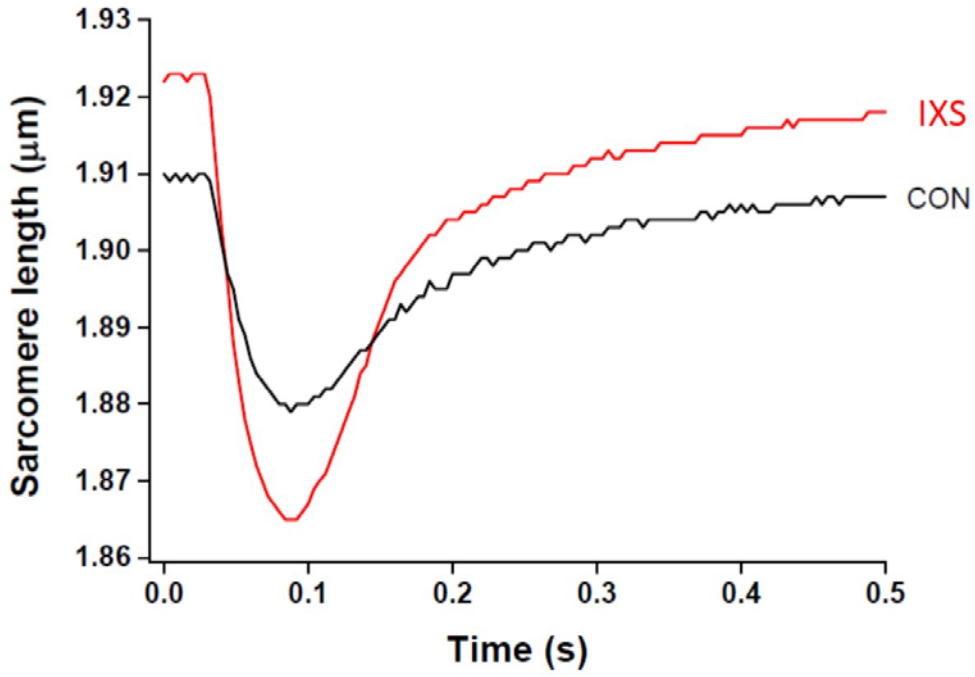


Figure 7.2 A single contraction and relaxation cycle in control (Con) and IXS.

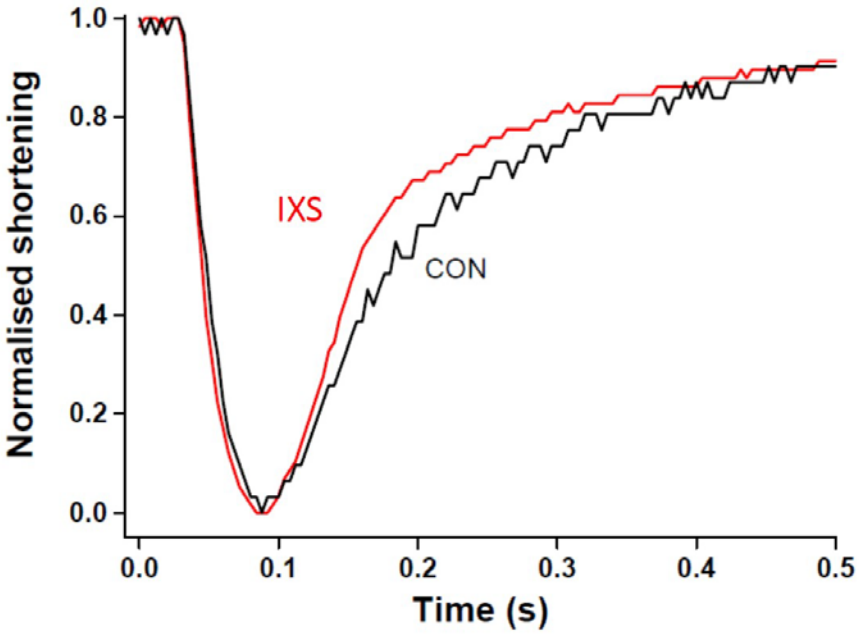


Figure 7.3 Single contraction with normalised shortening to demonstrate speed of relaxation in IXS and Control.

**Change in mechanical properties of cardiomyocytes on exposure to IXS**

Mean data for 26 cells from 8 animals are given in Table 7.1. Fig 7.4 gives the changes in SL (no of folds) during exposure to IXS compared to pre-exposure and after wash out. Exposure to IXS significantly lengthened diastolic SL and shortened systolic SL, thus the amplitude of contraction was significantly increased. IXS also caused a significant lusitropic effect, speeding relaxation. Upon wash out of IXS, inotropic and lusitropic effects reversed and SL shortening was significantly smaller than pre-exposure to IXS.

**Table 7.1** Effects of IXS on Isolated Cardiomyocytes

| | Con | IXS1 | IXS2 | IXS5 | Wash |
|---|---|---|---|---|---|
| Diastolic Sarcomere Length(μm) | 1.85±0.01 | 1.86±0.01$^{*\#\cmd}$ | 1.86±0.01$^{*\#}$ | 1.87±0.01$^{*\#}$ | 1.85±0.02 |
| Fractional Shortening (%) | 4.11±0.43 | 6.04±0.47$^{*\#\cmd}$ | 5.81±0.44$^{*\#}$ | 5.1±0.40$^{*\#}$ | 3.01±0.42$^{*}$ |
| Tau (ms) | 56.0±5.31 | 39.5±1.20$^{*\#}$ | 40.1±2.42$^{*\#}$ | 41.7±2.28$^{*\#}$ | 57.3±3.60 |

Mechanical properties of isolated cardiomyocytes stimulated at 1 Hz prior to exposure to IXS (Con); 1min (IXS1), 2min (IXS2) and 5min (IXS5) following exposure to IXS and 2min after return to IXS-free solution (Wash). IXS caused a significant increase in diastolic sarcomere length, increase in the amplitude of shortening and decrease in the time constant of relaxation (Tau). Repeated Measures ANOVA $P < 0.001$. Pairwise multiple comparisons *$P<0.05$ vs Con, $^{\#}P< 0.05$ vs Wash, $^{\cmd}P < 0.05$ vs IXS5. (n=26 cells from 8 animals).

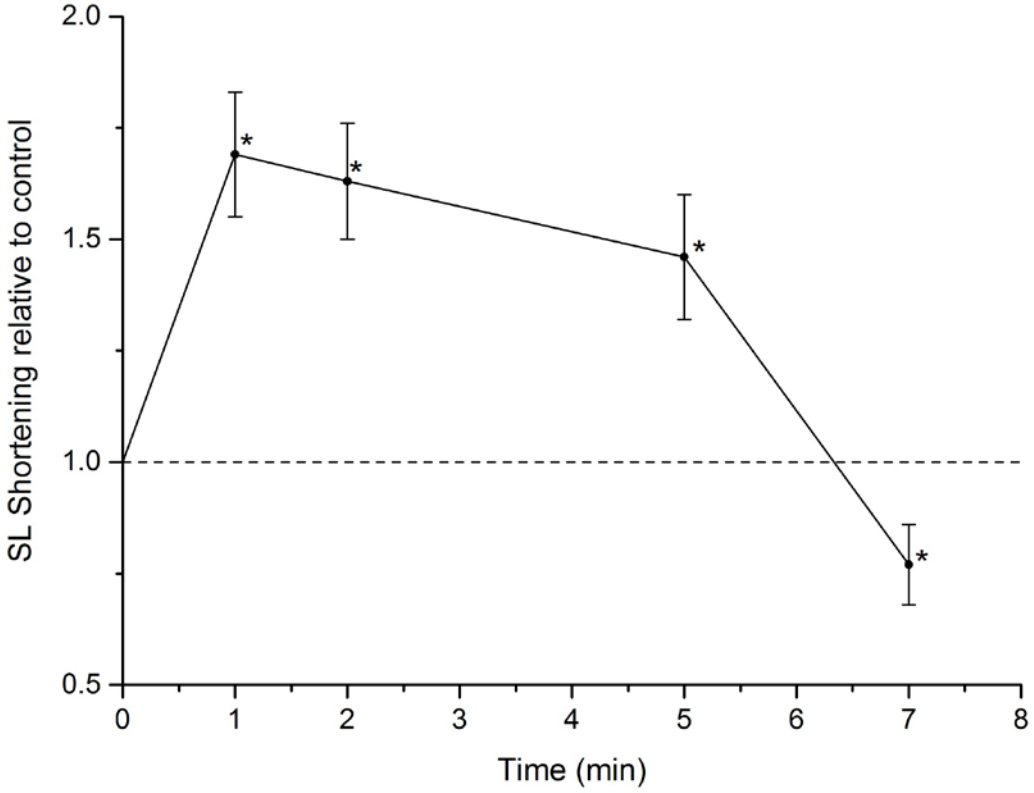


Figure 7.4 The proportional change in fractional shortening at 1min, 2min, 5min in IXS and 2 min after wash out compared to control at 0 min. *P<0.05 vs control (n=26 myocytes from 8 animals). Error bars show SEM.

### 7.3.2 Effect of IXS on the contraction of ventricular myocytes in the presence of Protein Kinase A (PKA) blocker

The changes in myocyte contraction caused by IXS were characteristic of PKA stimulation. Therefore, the original experiment was repeated using cells treated with the PKA inhibitor Rp8-Br-cAMPS (Rp8). Rp8 abolished the effect of IXS on fractional shortening e.g. in the absence of Rp8, after 2min in IXS, there was >60% increase in fractional shortening but in the presence of Rp8, there was a 0% change (Fig 7.5). The effects of IXS on diastolic SL (Fig 7.5) and relaxation (Fig 7.5) were also attenuated by Rp8 when compared to cells not treated with Rp8. The effect was such that in the presence of Rp8, IXS caused no significant change in fractional shortening, diastolic SL or speed of relaxation (Table 7.5 & Fig 7.5).

**Table 7.2** Effects of IXS on Isolated Cardiomyocytes treated with Rp8

| | Con | IXS1 | IXS2 | IXS5 | Wash |
|---|---|---|---|---|---|
| Diastolic Sarcomere Length(μm) | 1.88±0.01 | 1.88±0.01 | 1.88±0.01 | 1.88±0.01 | 1.87±0.01 |
| Fractional Shortening (%) | 8.43±0.78 | 9.29±0.77 | 8.41±0.75 | 7.29±0.90* | 5.20±1.00* |
| Tau (ms) | 54.5 ± 7.0 | 46.1±4.2 | 46.1±3.3 | 47.8±3.9 | 55.8±5.0 |

Mechanical properties of isolated cardiomyocytes, treated with Rp8, stimulated at 1 Hz prior to exposure to IXS (Con); 1min (IXS1), 2min (IS2) and 5min (IXS5) following exposure to IXS and 2min after return to IXS-free solution (Wash). In the presence of Rp8, the effects of IXS were abolished. Repeated measure ANOVA $p<0.05$. Pairwise multiple comparisons *$P<0.05$ vs Con. (n=13 myocytes, 3 animals).

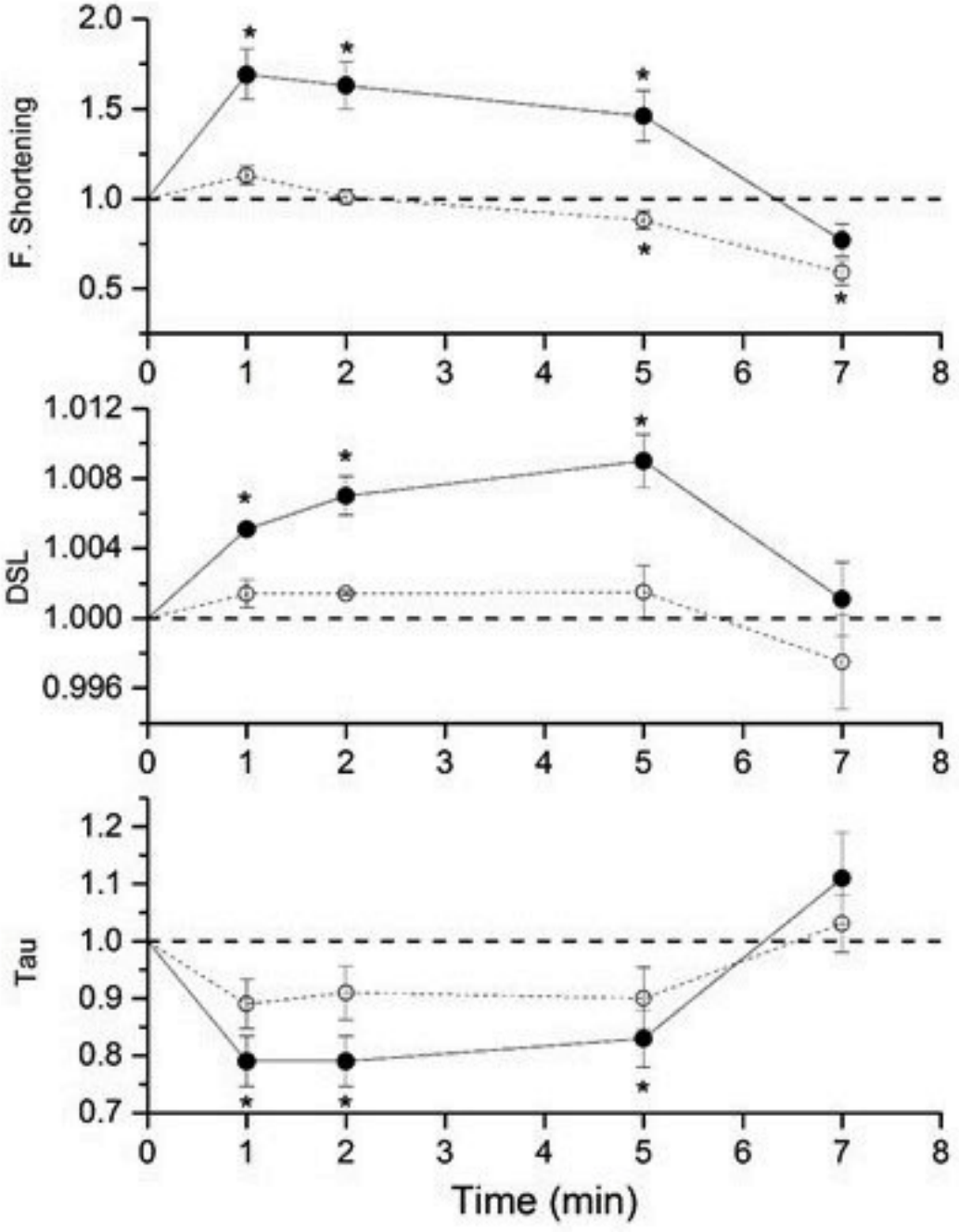


**Figure 7.5** Changes in fractional SL shortening, diastolic SL (DSL) and time constant of relaxation (Tau) in response to 5min exposure to IXS and 2min wash. (O) with and (●) without 30 min prior incubation with the PKA inhibitor, Rp8. Normalised values relative to those prior to IXS exposure at 0 min. The figure shows that the effects of IXS were significantly attenuated in cells treated with the PKA inhibitor, Rp8. *P<0.05 vs Control for each group (pairwise multiple comparisons). Error bars showing SEM. (n=13 myocytes from 3 animals)

### 7.3.3 Verification of action of Rp8

To verify the action of Rp8, cells were exposed to Isoprenaline (Iso) at a concentration of 10nmol/l in the presence or absence of Rp8 treatment. In the absence of Rp8, fractional shortening was increased from 7.34±2.08% in control to 11.18±1.91% on exposure to Iso ($P < 0.01$ vs control). In the presence of Rp8, there was only an increase in fractional shortening from 7.79±0.92% in control to 8.86±1.06% ($P < 0.05$ vs control). Thus, the effect of Iso was significantly attenuated by Rp8 (Fig 7.6).

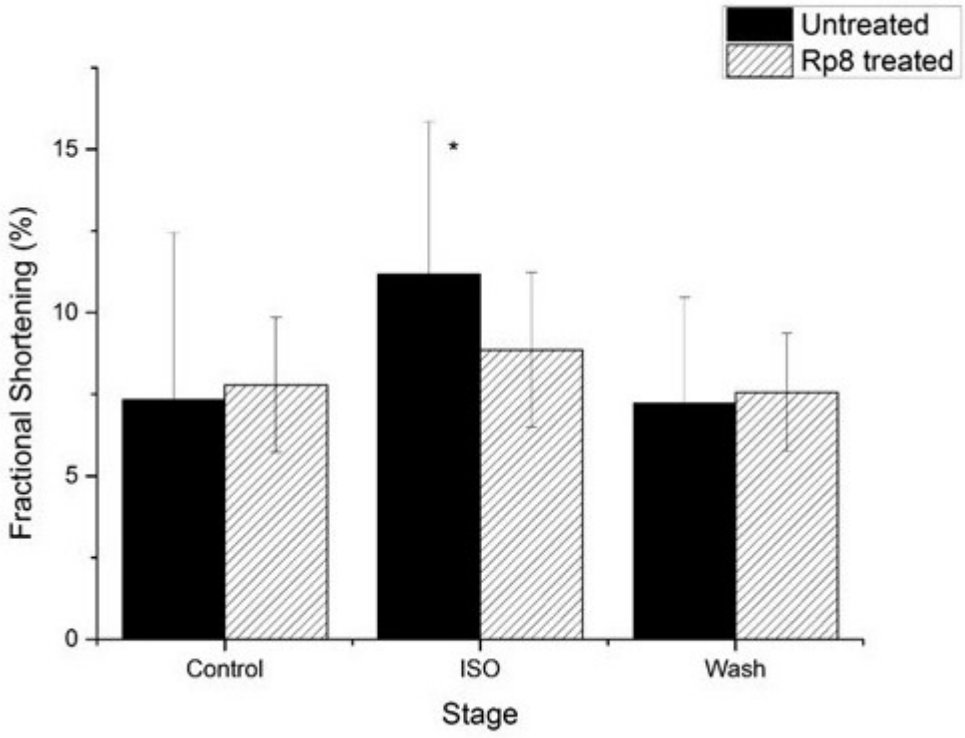


**Figure 7.6:** Changes in fractional shortening on exposure to isoprenaline (ISO) in cells treated with Rp8 and cells not treated with Rp8. Rp8 is shown to attenuate the positive inotropic effect of ISO. *P<0.05 on independent sample t-test vs Control.

### 7.3.4 Intracellular calcium transients

Although IXS caused a positive inotropic effect (e.g. Fig. 7.1&7.2) there was no corresponding increase in the $[Ca^{2+}]_i$ transient (Table 7.3).

**Table 7.3 Effect of IXS on intracellular $Ca^{2+}$ transients**

| | Con | IXS1 | IXS2 | IXS5 | Wash |
|---|---|---|---|---|---|
| Diastolic Calcium (RU) | 0.307±0.017 | 0.306±0.019$^{\#}$ | 0.307±0.020$^{\#}$ | 0.316±0.021 | 0.327±0.022* |
| Calcium transient amplitude (RU) | 0.130±0.019 | 0.126±0.019$^{\#}$ | 0.127±0.019 | 0.115±0.017* | 0.109±0.016* |

Characteristics of $[Ca^{2+}]_i$ transients of isolated cardiomyocytes stimulated at 1 Hz prior to exposure to IXS (Con); 1min (IXS1); 2min (IXS2) and 5min (IXS5) following exposure to IXS and 2 min after return to IXS-free solution (Wash). RU = ratio units. Repeated Measures ANOVA $P < 0.001$. Pairwise comparisons * $P< 0.05$ vs Con, $^{\#}$ $P < 0.05$ vs Wash. (n = 14 myocytes from 4 animals).

### 7.3.5 Fluorescence spectrophotometry

The effect of IXS on the fluorescent indicator, Fura-2 was characterised. Figure 7.7 (A) shows *in vitro* excitation spectra for Fura-2 at pCa 6 in the presence and absence of IXS. The absolute fluorescence in the presence of IXS was less than control, suggesting some quenching of fluorescence by IXS. The effect of IXS on the fluorescence ratio, our index of $[Ca2+]_i$, is shown in Figure 7.7 (B). The data (mean ± SEM, n = 4) were fitted to a logistic exponential curve. The fitted relationships suggest some flattening of the curve by IXS, though the measured ratios at each pCa were not significantly different from each other.

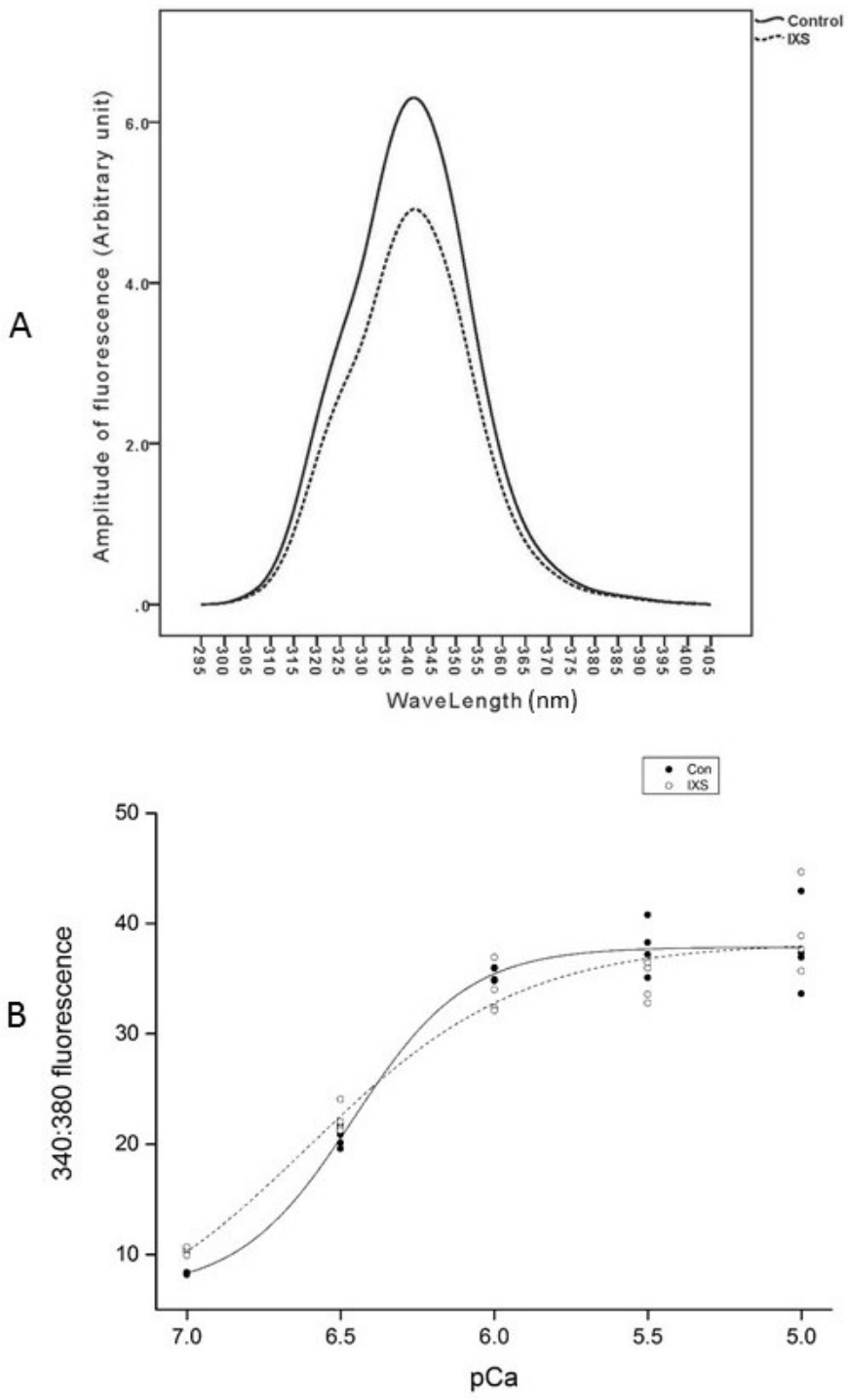


**Figure 7.7: A** Fluorescence spectra of Fura-2 free acid measured at 510 nm in response to excitation light between wavelengths 300nm and 400 nm at pCa6. The graphs show spectra in IXS (dotted line) and control (solid line) solution. The absolute fluorescence of Fura-2 was diminished by IXS. **B** 340nm/380nm ratio of Fura-2 in increasing calcium concentrations. The graph shows modified fitted relationships of the 340nm/380nm ratio to pCa in the presence of IXS (dotted line). n= 4 repeats in each solution.

## 7.4 Discussion

The hypothesis, that the uraemic toxin, IXS, would induce cardiodepressant effects on isolated mammalian cardiac myocytes was not supported by the experimental results. Instead, the results showed quite the opposite, that of acute positive inotropic and positive lusitropic effects on isolated cardiomyocytes.

The acute positive inotropic and lusitropic effects of IXS on cardiomyocytes seen in our study are similar to those seen following acute β-adrenoceptor stimulation[254,255] which also depend upon protein kinase A (PKA) activity and increased intracellular levels of cyclic adenosine monophosphate (cAMP). The observed increase in diastolic length is compatible with decreased myofilament $Ca^{2+}$ sensitivity as a result of PKA-dependent phosphorylation of troponin I[256,257] while the increased inotropy and lusitropy result from PKA-dependent phosphorylation of $Ca^{2+}$ handling proteins responsible for the entry of $Ca^{2+}$ into and removal from the cytosol of myocytes.[255]

The inhibitory effect of the PKA antagonist Rp8 on the effects of IXS suggests that IXS acts in a manner analogous to sympathomimetics and other positive inotropic agents that act upon the PKA-cAMP pathway, such as catecholamines and phosphodiesterase inhibitors.[258] The latter two have been shown to be deleterious when chronically present in heart failure (HF) patients through progressive HF and increased mortality.[259,260] The link between chronically activated sympathetic and renin-angiotensin-aldosterone (RAA) systems in established HF and cardiotoxicity of catecholamines[261] and angiotensin[207] is now recognized as the underlying mechanism responsible for progressive ventricular dysfunction in HF patients.[262] This in turn explains the long-term beneficial effects of inhibitors of renin-angiotensin-aldosterone and sympathetic systems in HF therapy.[211,212]

It would appear that chronic positive inotropic effects of IXS (and potentially other uraemic toxins) and eventual diminution of cardiac pumping capability in CKD patients mirror those of catecholamines and angiotensin in HF patients. Hence, the experimental findings reveal that the cardiotoxicity in uraemia parallels the pathophysiological mechanisms underlying cardiomyopathic processes seen in patients with primary cardiac failure.

## 7.5 Limitations

There was no increase in the amplitude of the Fura-2 fluorescence ratio on exposure to IXS. This seems incompatible with a PKA-mediated mechanism of action, given the well-documented increase in the $[Ca^{2+}]_i$ transient that underlies the positive inotropic effect of stimulating the cyclic AMP-PKA pathway in myocytes. The previous experiments in the lab with increasing extracellular $Ca^{2+}$ has predicted that one should have detected an approximately 16% increase in $[Ca^{2+}]_i$ transient amplitude for the observed increase in contraction caused by IXS, that is, within the sensitivity of the fluorescence system. However, *in vitro* spectrophotometry investigation showed that IXS quenches Fura-2 fluorescence and this may have influenced the *in vivo* measure of $[Ca^{2+}]_i$ Therefore, at present I interpret the lack of effect of IXS on the $[Ca^{2+}]_i$ transient with caution. An explanation of the positive inotropic effect of IXS based on increased myofilament $Ca^{2+}$-sensitivity does not seem credible given the longer diastolic SL and faster relaxation also observed in the presence of IXS.

IXS stock solution was tested for lipopolysaccharide contamination by a kinetic chromogenic limulus amoebyte lysate test (Lonza, Vervier, Belgium). Therefore, contamination is unlikely to be a reason for the observed effect of IXS.

## 7.6 Conclusion

In conclusion IXS has a direct effect on cardiomyocytes causing positive inotropy and lusitropy. IXS appears to act through PKA thus increasing the risk of cardiotoxicity on long-term exposure. Further research is needed to identify methods of reducing circulating levels of protein bound uraemic toxins. In addition, evaluating the effects of beta blockade in ameliorating uraemic cardiomyopathy may be beneficial in the future.

# CHAPTER 8

## Discussion, future directions and conclusions

# Discussion, future directions and conclusions

## 8.1 Discussion

Epidemiological studies in the 1990s[7,9] highlighted the enormous impact of cardiovascular disease morbidity and mortality in CKD. An astounding 50% of ESRD patients were shown to die of CVD.[263] Consequently, several research efforts across the globe tried to elucidate the underlying mechanisms of CVD in CKD. Hypertension, accelerated atherosclerosis, vascular calcification, anaemia, salt and water retention, and neurohumoral activation were all considered as viable mechanisms. However, interventions aimed at addressing the vasculopathic component of the *cardio-vascular* disease in CKD had at best shown only limited benefit.[30-32] Hence, studies evaluating the cardiomyopathic component were required. Indeed, this was highly pertinent as heart failure was shown to be the predominant cardiac disease of CKD and ESRD.[264] As a result, a report by KDIGO (Kidney Disease: Improving Global Outcome) in 2011 highlighted that evaluation of asymptomatic cardiac dysfunction is a research priority.[265] Although studies using imaging techniques have revealed the cardiac structural characteristics in asymptomatic patients, its functional correlate has not been as well studied. As the resting cardiac function in a failing heart is kept near normal by compensatory mechanisms, we measured the peak cardiac performance to reveal the underlying myocardial dysfunction.

The originality of the study can be summarised as follows –

1. Peak cardiac power and cardiac functional reserve have never been measured before in chronic kidney disease.

2. These are the first studies to test a CKD cohort without pre-existing cardiac disease or diabetes in relation to evaluating uraemic cardiomyopathy.
3. The differential role of cardiac output and tissue $O_2$ extraction in determining exercise capacity in CKD has not been shown in CKD in the past.
4. This is the first study to demonstrate the alterations in peak cardiac power and cardiac reserve with renal transplantation.
5. This is the first study to show the association between cardiac dysfunction and protein bound uraemic toxins.
6. The direct effects of indoxyl sulphate, a protein bound uraemic toxin, on the mechanical properties of isolated cardiomyocytes have been shown for the first time.

**Results of the studies**

**Study 1** describes the results of the cross-sectional study of CKD patients, control subjects and heart failure patients wherein peak cardiac power was measured along with several biochemical and haemodynamic variables.

> Hypothesis: Peak cardiac power and cardiac functional reserve are impaired in asymptomatic chronic kidney disease patients in the absence of any known cardiac diseases or diabetes mellitus.

I accept the hypothesis because the results demonstrated that peak cardiac power and cardiac reserve are impaired in asymptomatic CKD patients even in the absence of primary cardiac disease and diabetes. Moreover, the results showed a graded reduction in $CPO_{max}$ in CKD patients proportional to the severity of CKD.

The fact that the $CPO_{max}$ of CKD patients was shown to lie between that of healthy controls and heart failure patients is highly pertinent in understanding the mechanism of such cardiac dysfunction. The haemodynamic alterations in CKD mirrored that of heart failure patients with impaired pressure and volume generating capacities, impaired chronotropic reserve and impaired contractility. Thus, the

characteristics of heart failure were shown to be emerging in CKD even at an early asymptomatic stage. Although it is known that the mediators of progressive myocardial dysfunction in heart failure [vide infra] are also active in CKD, this is the first study to demonstrate the similarities in cardiac biomechanics between asymptomatic CKD patients and symptomatic heart failure patients.

The pathophysiological mediators with the potential to cause progressive myocardial dysfunction found in HF and CKD include renin angiotensin aldosterone system (RAAS) activation, sympathetic activation, pro-inflammatory cytokines and myocardial wall stress.[84,201-205] In heart failure, these mediators were shown to cause direct cardiomyocyte toxicity by inducing apoptosis and necrosis, and pathological hypertrophy resulting in progressive myocardial dysfunction.[115,118,206-209] The benefits of RAAS blockade and beta blockade in preventing the progression of myocardial dysfunction has been shown consistently[210-212] in heart failure and such treatments form the cornerstone of heart failure management. However, similar studies in CKD are still lacking. The existence of cardiomyopathy in CKD means that such studies are worthwhile in the future.

**Study 2** of the thesis is a longitudinal study of alterations in peak cardiac power and central haemodynamics with significant improvement in renal function with renal transplantation.

> Hypothesis: Renal transplantation improves peak cardiac power in chronic kidney disease.

Although it is a small study of 6 patients, all patients showed improvement in peak cardiac power and the increment resulted from improvement in pressure and volume generating capacities and the chronotropic reserve. Hence, I accept the above

hypothesis. The study demonstrated that the myocardial dysfunction of uraemic cardiomyopathy is potentially reversible. Further large longitudinal studies are required to understand the mechanism of such cardiac functional improvement.

**Study 3** of the thesis is a cross sectional study of cardiac structure in asymptomatic CKD patients using echocardiography. Although there were several such studies in the existing literature, the present study evaluated cardiac structure in asymptomatic subjects without confounding factors such as pre-existing heart disease or diabetes.

> Hypothesis: Cardiac structure is altered in asymptomatic chronic kidney disease patients in the absences of any known cardiac disease or diabetes mellitus.

Whereas Study 1 showed decreasing peak cardiac power with increasing severity of CKD, Study 3 showed increasing left ventricular mass with increasing CKD severity. Hence, I accept the hypothesis. Furthermore, such an inverse relationship is suggestive of ‘pathological hypertrophy’ or ‘maladaptive remodelling’ in CKD.

Decades of heart failure research has shown that pathological hypertrophy can lead to myocardial dysfunction and vice versa setting in motion a vicious cycle leading to progressive myocardial dysfunction.[118] It has also been shown that RAAS blockade and more importantly beta blockade can break the cycle and lead to *reverse remodelling* of the heart and restoration of myocardial function.[118] Further studies are needed to evaluate the benefits of such strategies in ameliorating uraemic cardiomyopathy.

**Study 4** is a cross sectional study evaluating the association between cardiac dysfunction and serum levels of protein bound uraemic toxins.

> Hypothesis: Serum protein-bound uraemic toxins are inversely related to peak cardiac power in chronic kidney disease.

The ability to measure subclinical cardiac dysfunction even in early stages of CKD enabled evaluation of its association with a wide range of serum levels of candidate uraemic toxins. Indoxyl sulphate and p-cresyl sulphate were found to be independently associated with cardiac dysfunction. More importantly the association was found with the physiologically relevant free concentrations of these toxins. Although the result does not prove a causality the present study is the first to demonstrate the association between cardiac dysfunction and IXS and PCS making these toxins suitable candidates for further *in vitro* studies evaluating the mechanism of cardiotoxicity.

**Study 5** describes the effect of indoxyl sulphate on the mechanical properties of isolated rat cardiomyocytes.

> Hypothesis: Indoxyl sulphate has direct inhibitory effect on mechanical properties of isolated rodent cardiomyocytes.

The experiments revealed that IXS has direct physiological effect on cardiomyocytes causing positive inotropy and lusitropy. Furthermore, it increased the resting diastolic fibre length. As these changes caused by IXS to the contractile properties were similar to that of protein kinase A (PKA) stimulation, further experiments were conducted with a PKA inhibitor. The results show that the effects of IXS were abolished by the PKA inhibitor suggesting that IXS acts through pathways involving PKA similar to other positive inotropic agents such as catecholamines and phosphodiesterase inhibitors.

Based on the results I reject the above hypothesis. However, the results still offer a plausible pathophysiological mechanism of myocardial dysfunction in CKD. The positive inotropic agents such as catecholamines and phosphodiesterase inhibitors have been shown to be deleterious when chronically present in heart failure (HF)

patients as they cause progressive HF and increased mortality.[259,260] It would appear that chronic positive inotropic effects of IXS (and potentially other uraemic toxins) and eventual diminution of cardiac pumping capability in CKD patients mirror those of catecholamines in HF patients.

In summary, measuring peak performance instead of the resting performance of the heart revealed a hitherto unknown asymptomatic cardiac dysfunction in CKD. This ability to quantify subclinical cardiac dysfunction helped explore its association with novel mediators of cardiotoxicity such as protein bound uraemic toxins in a wide range of CKD patients and helped identify putative cardiotoxins. Further *in vitro* experiments revealed a possible mechanism of cardiotoxicity of a candidate uraemic toxin. Thus, the thesis describes a 'reverse translational' research that revealed the existence of true cardiomyopathy in CKD and a possible mechanism for the evolution of such cardiomyopathy.

## 8.2 Future directions

Measuring peak performance of the heart has the unique advantage of revealing sub-clinical cardiac dysfunction thus providing a tool for studying early cardiac dysfunction in CKD. Furthermore, the tool can be applied in assessing cardiac functional benefits of various interventions. Some of the potential future applications of the present study are listed below.

1. Longitudinal follow up of the study cohort to determine whether $CPO_{max}$ predicts cardiovascular mortality and cardiovascular morbidity especially heart failure.
2. Longitudinal study of CKD patients before and after kidney transplantation measuring $CPO_{max}$, echocardiographic parameters of cardiac structure and function and biochemical parameters including protein bound uraemic toxins.
3. Longitudinal study evaluating the effect of other renal replacement therapy modalities such as haemodialysis and peritoneal dialysis on cardiac power and cardiac reserve.
4. Evaluation of the benefits of pharmacological intervention such as beta blockers in effecting cardiac *'reverse remodeling'* in uraemic cardiomyopathy.
5. Evaluation of the effect of exercise training on cardiac power, cardiac output, tissue $O_2$ extraction and peak $O_2$ consumption in CKD.
6. Further *in vitro* studies to assess the cardiotoxicity of other uraemic toxins. The effect of uraemic toxins on both mechanical and electrical properties can be studied using isolated cardiomyocytes and whole heart preparations.

## 8.3 Conclusion

The research studies described in this thesis demonstrate that asymptomatic cardiac dysfunction, the precursor of symptomatic heart failure, is present even in early CKD and even in the absence of any pre-existing heart disease or diabetes. Further in-depth evaluation of central haemodynamics and cardiac biomechanics reveal the similarities between CKD and established heart failure highlighting the existence of true cardiomyopathy in CKD. By showing the concomitant presence of left ventricular hypertrophy and myocardial dysfunction, the studies suggest the presence of 'pathological remodelling' of the heart in CKD. In addition, the reversal of this subclinical cardiac dysfunction after renal transplantation has also been demonstrated. The studies also offer further mechanistic insights by demonstrating the association between protein bound uraemic toxins and cardiac dysfunction *in vivo* and a mechanism of cardiotoxicity *in vitro*. Cardiorenal studies so far have largely focused on the vasculopathic processes and our study highlights the existence of the cardiomyopathic component in the pathophysiology of the cardio-vascular disease of CKD and thereby emphasises the need for therapeutic strategies addressing such a defect.

# Chapter 9

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