DOCTOR OF PHILOSOPHY

Physical activity, kidney function and kidney injury

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PHYSICAL ACTIVITY, KIDNEY FUNCTION AND KIDNEY INJURY

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SUMMARY OF FINDINGS

This PhD sought to exploit the acute effects of exercise upon the kidneys to make tenable links to pathological states such as acute kidney injury (AKI) and chronic kidney disease (CKD). It is surprising that such associations with their potential clinical implications have received limited attention so far despite the ever-increasing number of healthy individuals participating in vigorous and physiologically challenging activities. The work herein has shown how experimental *in-vivo* exercise models may be used to simulate a "stressed" kidney with features that resemble diseased states.

Summarising the key findings briefly, the first study (**chapter 2**) demonstrated that maximal-intensity exercise in the form of an 800 metre sprint resulted in increased urinary concentrations of an AKI biomarker (neutrophil gelatinase-associated lipocalin / NGAL), suggesting mild kidney stress or a concentrating effect. However, plasma NGAL concentrations decreased and urinary rises were independent of post-exercise proteinuria. There was also an inverse relationship between urinary volume and urinary NGAL concentrations – an observation that is also seen in oliguric AKI.

The systematic review in the second study (**chapter 3)** found promise in post-exercise proteinuria as a predictor for CKD progression. Five studies (N = 351) that met inclusion criteria, examined prospective cohorts of Type I diabetics who were at risk of CKD. Through combining the results of the primary outcome in four studies (N = 318), the presence of post-exercise proteinuria was highly associated with elevated resting proteinuria at follow-up (χ^2 test, $P < 0.0001$) and significant odds ratios (developing CKD following a positive exercise test vs. not developing CKD) were noted in each of these four studies (OR 2.3-52.0). However, there was great variability and questionable validity in the interventions that did not permit meta-analysis. It was evident that exercise interventions need to be refined and standardised before applying to other at-risk CKD populations.

In the third study (**chapter 4)**, it was demonstrated that a prior bout of muscle-damaging exercise established a pro-inflammatory state with elevated plasma interleukin-6 (IL-6) concentrations, and that with subsequent endurance-based exercise in the heat there was increased kidney stress as measured by increased urinary NGAL and plasma creatinine concentrations. The latter elevations met clincial criteria for AKI. Also, plasma IL-6 and plasma NGAL concentrations were positively correlated.

Lastly, the final study (**chapter 5)** extended the findings of chapter 4 by isolating the role of pro-inflammatory IL-6 in AKI. Through infusion of recombinant IL-6 in healthy males to concentrations above 100 pg/ml, elevations in plasma NGAL concentrations were shown but not to AKI ranges. In addition, there were no changes to plasma concentrations of other AKI biomarkers such as creatinine or cystatin C. Overall, this suggests that IL-6 is able to modulate NGAL but is not responsible *per se* for AKI or kidney dysfunction. Thus, it is likely that additional physiological aberrations are needed.

This work has been published and presented in the following peer-reviewed journals and international conferences:

Chapter 2

Junglee NA, Lemmey AB, Burton M, Searell C, Jones D, Lawley JS, Jibani MM, Macdonald JH (2012). Does proteinuria-inducing physical activity increase biomarkers of acute kidney injury? *Kidney Blood Press Res* **36**, 278-289.

Junglee NA, Lemmey AB, Burton M, Searell C, Jones D, Lawley JS, Jibani MM, Macdonald JH The response of neutrophil gelatinase-associated lipocalin to proteinuria-inducing physical activity: an observational prospective cohort study. Poster presentation at British Renal Society 2012 conference, Manchester, UK.

Chapter 3

Junglee NA, Jibani MM, Lemmey AB, Macdonald JH. Can exercise-induced proteinuria predict the onset or progression of chronic kidney disease? A systematic review. Poster presentation at the American Society of Nephrology Kidney Week 2015, San Diego, USA.

Chapter 4

Junglee NA, Di Felice U, Dolci A Fortes MB, Jibani MM, Lemmey AB, Walsh NP, Macdonald JH (2013). Exercising in a hot environment with muscle damage: effects on acute kidney injury biomarkers and kidney function. *Am J Physiol Renal Physiol* **305**, F813-820.

Fortes MB, Di Felice U, Dolci A, Junglee NA, Crockford MJ, West L, Hillier-Smith R, Macdonald JH, Walsh NP (2013). Muscle-damaging exercise increases heat strain during subsequent exercise heat stress. *Med Sci Sports Exerc* **45**, 1915-1924.

Fortes MB, Di Felice U, Dolci A, Junglee NA, Crockford MJ, West L, Hillier-Smith R, Macdonald JH, Walsh NP. Exercising in a hot environment with muscle damage: effects on acute kidney injury biomarkers and kidney function. Oral abstract presentation (AKI clinical studies I) at the American Society of Nephrology 2012 Kidney Week, San Diego, USA.

Chapter 5

Junglee NA, Searell CR, Jibani MM, Macdonald JH (2014). Experimental acute kidney injury. *Nephrol Dial Transplant* **29,** iii90-iii101 [abstract].

Junglee NA, Searell CR, Jibani MM, Macdonald JH. Infusion of recombinant IL-6 in healthy humans elevated plasma NGAL concentrations without a reduction in renal function. Poster presentation at the 51st European Renal Association congress 2014, Amsterdam, Netherlands.

LIST OF CONTENTS

INDEX

LIST OF TABLES

with no microalbuminuria on fixed heart rate. [d] Unable to determine number who developed PeP as no cut-off was defined. [e] Authors split cohort into seven groups depending on their exercise and overnight albumin excretion rate values. Only group 6 exhibited PeP and elevated resting albumin excretion rate on follow-up. See Garg *et al*. (1990) for further details. [f] Authors considered PeP and rest A:Cr as >4.3 and >2.1 mg/mmol as equivalent to >30 ug/min and 15 ug/min, respectively. [g] Follow-up on focussed on PeP cohort's results. [h] Where there was 0 participants in a category for calcuation of odds ratio, this was replaced by 0.5 (Glas *et al.*, 2003). NS, not specified; PeP, post-exercise proteinuria; A:Cr, albumin-to-creatinine ratio.

Table 3.4. Bias assessment using QUADAS-2 tool for all studies meeting criteria for systematic review. Extra signalling questions were included (see appendices 2 and 3 for elaboration). Green boxes denote low risk of bias/concerns regarding applicability. Red boxes denote high risk of bias/concerns regarding applicability. N, no; Y, yes; U, unsure.

Table 3.5. Some of the factors that should be considered when developing an exercise test for proteinuria provocation.

CHAPTER 4 - EXERCISING IN A HOT ENVIRONMENT WITH MUSCLE DAMAGE: EFFECT ON ACUTE INJURY BIOMARKERS AND KIDNEY FUNCTION

Table 4.1. Kidney function markers following exercise induced muscle damage or control exercise. Legend: Data are mean ± SD, and mean difference between groups [95% confidence interval]; EIMD, exercise-induced muscle damage; CON, control; by main effect: *, significantly different from before heat stress; †, significantly different from CON.

Table 4.2. Treatment responses following exercise induced muscle damage or control exercise. Legend: Data are mean ± SD, and mean difference between groups [95% confidence interval]; EIMD, exercise-induced muscle damage; CON, control; by main effect or by *post-hoc* test following significant interaction: *, significantly different from before heat stress; †, significantly different from CON.

CHAPTER 5 – INFUSION OF RECOMBINANT IL-6 IN HEALTHY HUMANS ELEVATES PLASMA NGAL CONCENTRATIONS WITHOUT A REDUCTION IN RENAL FUNCTION.

Table 5.1. Effect of recombinant IL-6 infusion upon heart rate, blood pressure and tympanic temperature at 0 hours and 3 hours. Legend: Data are mean ± SD except for systolic blood pressure (median {interquartile range}). *, significantly different from 0 hours by paired t-test.

LIST OF FIGURES

CHAPTER 1 - GENERAL INTRODUCTION

Figure 1.5. There a strong positive correlation (r = 0.95; $P < 0.0001$) between spot urine protein-to-creatinine (Pr/Cr) ratio and concurrent 24-hour urine protein excretion as demonstrated in 174 adult patients with chronic kidney disease. Similar findings have been noted in many other studies and consequently use of protein-to-creatnine ratio has supplanted the use of 24-hour proteinuria estimations in routine clinical and research settings. Adopted from Siu *et al.,* 2011.

Figure 1.6. Mean albuminuria excretion rates at different absolute intensities of exercise as measured by running distance. Grey bars represent mean values at resting state. Black bars are mean values following exercise. A 800 metre sprint has the highest propensity for post-exercise proteinuria. This progressively falls as longer distances are encountered. However, very short-term exercise (e.g. 100 metre sprint) has a lesser effect than longer runs. Adapted from Poortmans, 1994.

Figure 1.7. Comparison between the molecular weight of proteins and their renal clearance under normal resting conditions (filled bars) and post-exercise (unfilled bars). The letter under each pair of columns indicates the plasma protein studied: A, β_2-glycoprotein I; B, α_1-acid glycoprotein; C. α_1-antitrypsin D, Gc-globulin; E, tryptophan-rich prealbumin; F, albumin; G, hemopexin; H, transferrin; I, haptoglobin; J, cerulplasmin; K, γA-globulin; and L, γG-globulin. It is clear that proteins of a higher molecular weight are excreted following exercise suggesting an increase in glomerular permeability. Adopted from Poortmans and Jeanloz, 1968.

Figure 1.8. Examples of normal and dysmorphic erythrocytes as seen by phase-contrast microscopy (at x400 magnification): A) normal erythrocyte; B) ghost erythrocyte (where haemoglobin has leached out); C) dysmorphic erythrocyte extruding small bleb from membrane; D) dysmorphic erythrocyte with ruptured membrane. Adopted from Pillsworth Jr *et al.*, 1987.

Figure 1.9. Urine samples taken during a seven-mile run in an individual prone to march haemoglobinuria. Tubes 1, 2 and 3 represent urine taken before, 20 and 60 minutes after running with insoles, respectively. Tubes 4, 5 and 6 represent the same time points of urine sampling, but taken whilst running without insoles. With the insoles there was no detectable change in plasma haemoglobin or haptoglobin. Adopted from Buckle, 1965.

Figure 1.10. Cardiac troponin T measured in individual competitors following the London Marathon in 2005. Approximately 78% of runners demonstrated troponin T values above 0.01 μg/l, 58% above 0.03 μg/l, 36% above 0.05 μg/l and 11% above 0.1 μg/l. All cut-offs have been diagnostic of an acute myocardial infarction. cTnT, cardiac troponin T. Adopted from Shave *et al.*, 2005.

Figure 1.11. Transthoracic echocardiogram images in a marathon participant with a troponin-T concentration that increased from 0.01 to 0.54 μg/l post-marathon. *(A)* Baseline image demonstrating normal right ventricular structure and function. *(B)* Post-marathon evidence of mild right ventricular dilatation (right ventricular end-diastolic diameter 38 mm) and a decrease in right ventricular fractional area change of 35%. *(C)* Post-marathon cine-image in the horizontal long-axis view confirming a decrease in the right ventricular ejection fraction of 44%. LA: left atrium; LV: left ventricle; RA: right atrium; RV: right ventricle. Adopted from Mousavi *et al.*, 2009.

Figure 1.12. One of the seminal studies demonstrating the clinical utility of NGAL as an AKI biomarker in children who underwent open-heart surgery with cardiopulmonary bypass. In the 20 patients who sustained AKI (N = 20), serum NGAL concentrations were elevated at two hours ($P = 0.001$) whereas those without AKI (N = 51) did not demonstrate any discernable rise ($P > 0.05$). Those with AKI had serum creatinine elevations (> 50% from baseline) 22 hours after NGAL. Adopted from Mishra *et al.*, 2005.

Figure 1.13. Kinetics of plasma myoglobin and creatine kinase (CPK) elevations following rhabdomyolysis. Myoglobin is the first protein to increase, but owing to its rapid clearance from plasma concentrations, rapidly return to normal within 24 hours. Conversely, CPK is elevated a few hours later than myoglobin but reaches its peak value within the first 24 hours and can remain as such for up to three days. This characteristic enables CPK to be a more practical marker for the severity and evolution of muscle injury. Adopted from Giannoglou *et al.*, 2007.

Figure 1.14. A proposed mechanism elucidating the complex relationship between vasoactive mediators, inflammatory responses and renal haemodynamics in exertional heat stroke. NO: nitric oxide; PG, prostaglandin; GFR, glomerular filtration rate; ARF, acute renal failure i.e. AKI. Adopted from Lin *et al.*, 2003.

Figure 2.5a. Effect of an 800 metre run upon urinary NGAL:creatinine ratio. Data are medians (thick lines) and interquartile ranges (boxes). Open circles and asterixes are outliers. There was a significant effect of time (P = 0.002). #, difference compared to pre-exercise sample point by *post hoc* test.

Figure 2.5b. Individual plots for urinary NGAL:creatinine ratio following an 800 metre run.

Figure 2.6. Effect of 800 metre run upon plasma NGAL level over time. For the sake of consistency, data are presented as boxplots whereas data in results section are mean (±SD). Data are medians (thick lines) and interquartile ranges (boxes). Open circles are outliers. Dotted line indicates upper limit of normal range for urinary NGAL. There was a significant effect of time (P < 0.002). #, difference compared to pre-exercise sample point by *post hoc* test.

CHAPTER 3 - CAN EXERCISE-INDUCED PROTEINURIA PREDICT THE ONSET OR PROGRESSION OF CHRONIC KIDNEY DISEASE IN HUMANS? A SYSTEMATIC REVIEW.

Figure 3.1. Flowchart used to select papers into systematic review. Specific values for definition of post-exercise proteinuria and primary outcomes are detailed in the *Eligibility Criteria* section. Inclusion of secondary outcomes were not an essential prerequisite for study inclusion. CKD: chronic kidney disease.

Figure 3.2. Flowchart detailed results of electronic database searches with reasons for exclusion at each stage.

Figure 3.3. Bar graphs summarising risk of bias and concerns of applicability across studies included for systematic review following assessment with QUADAS-2 tool. Green boxes denote low risk of bias/concerns regarding applicability. Red boxes denote high risk of bias/concerns regarding applicability.

Figure 4.1. Schematic detailing flow of study. Treatment (EIMD or CON) was performed at 20 ^{o}C. Heat stress was performed at 33 ^{o}C. Baseline, before heat stress and after heat stress denote sampling time points. EIMD, exercise-induced muscle damage arm; CON, control arm; NGAL, neutrophil gelatinase-associated lipocalin.

Figure 4.2. Left panel: urinary neutrophil gelatinase-associated lipocalin (NGAL) at baseline, before (preHS) and after (postHS) heat stress for control (CON; clear boxes) and exercise induced muscle damage (EIMD; shaded boxes) groups. Data are presented as median (solid line in box), quartiles (boxes) and ranges (whiskers) given non-parametric data. Circles and small stars indicate outliers. Dotted horizontal line on y-axis indicates upper limit of normal range for urinary NGAL (9.8 ng/ml). For EIMD and CON groups, there was a significant interaction between pre- and postHS (P = 0.005). By post hoc test: *, significantly different from PreHS; †, significantly different from CON. Right panel: median difference and 95% confidence intervals between EIMD and CON for urinary NGAL at pre- and postHS. A positive value indicates urinary NGAL was higher in the EIMD group.

Figure 4.3. Left panel: plasma neutrophil gelatinase-associated lipocalin (NGAL) at baseline, before (preHS) and after (postHS) heat stress for control (CON; dotted line) and muscle damage (EIMD; solid line) groups. Data are means (SD). Dotted horizontal grey line crossing y-axis indicates upper limit of plasma NGAL (106 ng/ml). For EIMD and CON groups, there was a significant interaction between pre- and postHS (P = 0.04). By post hoc test: *, significantly different from PreHS; ‡ trend ($P \geq 0.05$ but ≤ 0.1) for difference with CON. Right panel: mean difference and 95% confidence intervals between EIMD and CON for plasma NGAL at pre- and postHS. A positive value indicates plasma NGAL concentration was higher in the EIMD group.

Figure 4.4. Left panel: plasma creatinine at baseline, before (preHS) and after (postHS) heat stress for control (CON; dotted line) and muscle damage (EIMD; solid line) groups. Data are means (SD). Dotted horizontal grey line crossing y-axis indicates upper limit of plasma creatinine (1.2 mg/dl). For EIMD and CON groups, there was a significant interaction between pre- and postHS (P = 0.005). By post hoc test: *, significantly different from PreHS;

†, significantly different from CON. Right panel: mean difference and 95% confidence intervals between EIMD and CON for plasma creatinine at pre- and postHS. A positive value indicates plasma creatinine was higher in the EIMD group.

CHAPTER 5 – INFUSION OF RECOMBINANT IL-6 IN HEALTHY HUMANS ELEVATES PLASMA NGAL CONCENTRATIONS WITHOUT A REDUCTION IN RENAL FUNCTION.

Figure 5.1. Schematic illustrating time points for primary and secondary measurements. The recombinant IL-6 infusion was given between 0 and 3 hours (h). All measurements were taken in the resting condition.

Figure 5.2. Effect of recombinant IL-6 infusion upon plasma IL-6 concentrations. Data are medians (thick lines) and ranges. Open circles and asterixes are outliers. There was a significant effect of time (P = 0.007). #, difference compared to 0 hours sample point by planned comparisons.

Figure 5.3. Effect of recombinant IL-6 infusion upon plasma NGAL concentrations. Data are means and standard deviations. Dotted line indicates upper limit of normal range for plasma NGAL (106 ng/ml). There was a significant effect of time (P = 0.025). #, difference compared to 0 hours sample point by planned comparisons.

CHAPTER 6 - SUMMARY OF STUDIES, GENERAL DISCUSSION AND FUTURE DIRECTIONS

Figure 6.1. When considering urinary biomarker:creatinine ratios, the change of excretion of both biomarker and creatinine should be known. As creatinine excretion is unstable in AKI, changes in the ratio cannot be determined unless the directional change of each is known. This would also apply to other dynamic setting such as post-exercise. AKI, acute kidney injury**;** Δ**,** change**.** Adapted from Goldstein 2010.

Figure 6.2. Immunohistochemistry results on frozen sections of mouse kidneys at 3 and 12 hour reflow periods following experimentally-induced ischaemia using a vascular clamp. Induction of mouse kidney NGAL protein within tubules is demonstrated by cherry-red fluorescence when tissue samples were probed with a polyclonal antibody to NGAL. Con, representative sample obtained from control mice; G, glomerulus; HP, tissue sample at high

power 100× magnification (other panels are at 20× magnification). Adopted from Mishra *et al.*, 2003.

Figure 6.3. Arterial IL-6 concentrations at rest and during exercise during one hour on a cycle ergometer at a core temperature of 38^0C (first bout) followed by a one hour rest period and then further exercise at 39.5^0C (second bout). Both exercise trials were completed at a workload (170 ± 4 W) that corresponded to 50 % $\dot{V}O_{2max}$. A similar pattern of plasma IL-6 rise was also seen in study 4. Data are means of 8 subjects. * Significantly different from the resting value ($P < 0.05$). Adopted from Nybo *et al.* 2002.

Figure 6.4. Changes in serum creatinine in fourteen male athletes before and at several time points following a 50km dualthon. The cohort was split into a damaged group (N = 7) consisting of individuals with renal tubular epithelium in their urine and a non-damaged group (N = 7) where this was absent. Damaged individuals met stage I AKIN AKI criteria immediately post-exercise and 90 minutes post-exercise based upon serum creatinine concentrations elevations (0.54 mg/dl or 1.66-fold increase from baseline and 0.36 mg/dl or 1.44-fold rise from baseline, respectively). Legend: creatinine (Cr), pre-exercise (Pre), immediately post-exercise (0 h), 1.5 hour post-exercise (1.5 h) and 3 hour post-exercise (3 h) are sampling points. Statistics: * $P < 0.05$, ** $P < 0.01$, † $P < 0.1$. Adopted from Sugama *et al.* 2013.

Figure 6.5. Taylor *et al.* used a custom MR-compatible stationary cycle in a 0.5-T open magnet magnetic resonance scanner with cine phase-contrast techniques to measure blood flow velocities in the inferior vena cava of young healthy subjects at rest and during upright dynamic lower limb exercise. Subjects were strapped to an upright seat in the open magnet to allow full range of leg motion while positioning the abdomen in the centre of the magnet for optimal imaging. The cycle was then positioned and its resistance was adjusted to promote comfortable pedaling and minimize abdomen movement that could affect image capture and quality. Adopted from Taylor *et al.* 2002.

Figure 6.6. Schematic illustrating the potential mechanism of acute kidney injury through heavy physical exertion. In this setting, kidney injury is likely to exist on a scale of severity where milder activity only results in temporary phenomena such as haematuria and proteinuria. However, more strenuous activity of a higher intensity and longer duration,

coupled with various adverse modifiable and non-modifiable factors increases the risk of developing significant AKI. It is at this point where “normal” physiological responses to exercise cross-over into a clinically-significant pathological state. AKI, acute kidney injury; NSAID, non-sterodial anti-inflammatory drug.

Figure 6.7. Diagram summarising the key putative pathophysiological connections in the cardiorenal syndrome. Given the recent formal recognition of this syndrome, these potential routes of communication are now being investigated in more detail. RAAS, renin–angiotensin–aldosterone system; VD/VDR, vitamin D/vitamin D receptor. Adopted from Darabian *et al*. 2011.

COMMONLY USED ABBREVIATIONS

A:Cr albumin:creatinine ratio

ADH - anti-diuretic hormone

AKI - acute kidney injury

AKIN - acute kidney injury network

ANP - atrial natriuretic peptide

CKD – chronic kidney disease

Cr - creatinine

CON – control (group)

ELISA - Enzyme-linked immunosorbent assay

EIMD - exercise induced muscle damage (group)

GFR - glomerular filtration rate

IL- interleukin

NGAL - neutrophil gelatinase-associated lipocalin

NGAL:Cr - neutrophil gelatinase-associated lipocalin: creatinine ratio

P:Cr – protein:creatinine ratio

PeP – post-exercise proteinuria

RBF – renal blood flow

RPF – renal plasma flow

TNF-α – tumour necrosis factor alpha

$\dot{V}O_{2max}$ – maximal aerobic capacity

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AUTHOR'S DECLARATION AND CONSENT

The author of this PhD thesis has contributed to the following aspects of each study as described in chapter 2 to 5.

	Chapter 2	Chapter 3	Chapter 4	Chapter 5
Conception and design	✓	✓	✓	✓
Data collection	✓	✓	✓in part [2]	✓in part [3]
Stastistical analysis	✓	✓	✓	✓
Analysis and interpretation	✓	✓in part [1]	✓	✓
Writing paper	✓	✓	✓	✓
Critical revision of paper	✓	✓	✓	✓
Final review of paper	✓	✓	✓	✓

[1] Analysis and interpretation of systematic review was done in conjunction with Dr Jamie Macdonald.

[2] Data collection was done collectively as part of a larger research group.

[3] Samples were collected from staff at the Centre of Inflammation and Metabolism, Denmark.

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FORMAT OF PHD THESIS

Chapter 1 of this thesis begins with a detailed discourse into the numerous changes to kidney physiology with exercise and ends by discussing how such observations may develop into pathological states. This literature review covers research finding upto and including December 2011 (end of 1st year PhD). Chapters 2, 4 and 5 detail experimental work based upon specific questions raised by the introduction. Chapter 3 is a systematic review. All the studies in chapters 2 to 5 are arranged in a paper-based format. Chapter 6 concludes the thesis with a general discussion highlighting important findings from the preceding chapters, recent literature (upto and including May 2015), and some of their clinical implications.

The layout of this thesis is in accordance with recommendations in the Bangor University PhD Student Handbook 2012.

CHAPTER 1

GENERAL INTRODUCTION

Compared to the vast scientific literature on cardiac responses during exercise, the kidneys have been relatively ignored. However, their contribution to the maintenance of homeostasis in the setting of strenuous exertion cannot be underrated. For example, they are essential to the diversion of blood flow to active musculature, maintainance of intravascular volume by limiting filtration processes, and regulating electrolyte concentrations, particularly sodium and potassium (Grimby 1965a; Refsum and Strömme 1975; Zambraski 1990).

These and other concomitant physiological changes such as reduced kidney blood flow, reduced glomerular filtration rate (GFR) and the presence of blood and protein in the urine (haematuria and proteinuria, respectively), could also be described as stress phenomena (Poortmans 1984, Poortmans and Vanderstraeten, 1994). The incidences of these vary and range from 11-100% depending on various factors that are described below (Bellingheri *et al.*, 2008).

Clinicians encounter such phenomena in the context of acute and chronic kidney disease (CKD) and these often serve as biomarkers of disease severity. In the acute setting a rapid progressive glomerulonephritis is characterised by a reduction in glomerular filtration and increased amounts of haematuria and proteinuria (Lewington 2008). Proteinuria is thought to be mechanistically important in CKD as it can directly contribute to further glomerular damage partly through activation of pro-inflammatory pathways (Abbate *et al.*, 2006). However, when these phenomena present during and following exercise they are, in the majority of cases, not as profound and are short-lived (hours to days) (Poortmans 1984; Bellingheri *et al.*, 2008). Consequently, physiologists and clinicians currently regard such alterations as harmless.

Nevertheless, there are numerous anecdotal reports in the literature that have associated strenuous exercise with significant acute disruption in kidney function (cf. acute kidney injury), through conditions such as heat stroke and rhabdomyolysis. The precise incidence of this is unknown but estimates of prevalence have approached 30% when strenuous exercise is performed in adverse environmental conditions e.g. in military personnel (MMWR 1990; Bosch *et al.*, 2009; MSMR, 2012). Usually individuals who succumb to disrupted kidney function with exercise are healthy and disease-free, but hospitalisation is often required and despite supportive treatments, there is a high risk of death or permanent disability (Sithinamsuwan *et al.*, 2009).

Thus, such phenomena that are shared between what are considered to be a physiological response from the kidneys and kidney disease, provides the impetus to investigate the potential mechanisms and risk factors that can convert healthy exercising individuals to a

state of ill health. Aside from furthering knowledge of kidney physiology during exercise, the findings gained from such endeavours could have important public health implications. For example, is heavy exercise "harmful" for the kidneys in certain individuals? Already such concepts have been investigated in other organs e.g. exercise, cardiac function and troponin elevations (Shave *et al.*, 2010). Additionally, any findings will be of interest to specific "at risk" populations who perform regular strenuous physical activity such as amateur and professional athletes, the military, and sectors of the public services (e.g. fire fighters). They will also provide a platform for further research in patients with established CKD, for whom exercise is currently being promoted as part of management strategy with little regard for its impact upon kidney function (Heiwe *et al.*, 2011).

Prior to focussing upon the specific circumstances in which strenuous exercise can result in significant kidney dysfunction, it is essential to provide a detailed description of the key physiological alterations that occur to the human kidneys during exercise, and a criticial appraisal of their measurement. In so doing, the reader can then perceive how such alterations may lead to a diseased state.

1.1. *PHYSIOLOGICAL CHANGES TO KIDNEY FUNCTION DURING EXERCISE*

1.1.1. *Kidney blood flow.* Measurement of actual or *real-time* renal (kidney) blood flow (RBF) in humans is technically challenging – even under normal resting conditions. This is complicated further during dynamic situations such as exercise owing to body movement. Non-invasive imaging techniques such as ultrasound Doppler have been used to demonstrate a general reduction in blood flow to all abdominal viscera during exercise with re-distribution to active musculature (Osada *et al.*, 1999). However, more specific measurements in each of the two renal arteries with this modality have proved to be difficult because of interference from intestinal gas (Delahunt *et al.*, 1996). Additionally, there are other technical limitations, including anatomic variations between subjects, interference from subcutaneous fat tissue and operator bias. Other imaging techniques such as nuclear magnetic resonance imaging have been used (Taylor *et al.*, 2002).

As an alternative, renal plasma flow (RPF) is closely related to RBF and can be mathematically defined and measured during and after exercise (Chapman *et al.*, 1948). If the arterial plasma (P_a), venous plasma (P_v) and urine (U_x) concentrations of a substance are known together with the urine flow rate (V), the following equation can be used:

$$RPF = \frac{U_x . V}{P_a - P_v}$$

This assumes that a non-metabolite entering the kidney via the renal artery will exit essentially intact through the renal vein and into urine via the ureters. Diodone and p-aminohippuric acid are chemically inert compounds that can be administered intravenously and utilised for this purpose (Chapman *et al.*, 1948; Philips and Hamilton, 1948; Wilson *et al.*, 1970). The latter is freely filtered and not reabsorbed within the nephron, though a small proportion in the *vasa recta* does not cross the Bowman's capsule. This fraction is reabsorbed and then secreted into the tubular lumen via the proximal convoluted tubule cells. Thus, an effective RPF can be calculated:

$$effective\ RPF = \frac{U_x}{P_a} . V$$

At rest, the kidneys receive approximately 20% of the total cardiac output (Guyton 1981). In comparison, skeletal muscles receive 20%, the liver, 30%, and 14% is delivered to the brain. With respect to blood flow, this is 4 ml/min/g in renal tissue compared to 0.04 ml/min/g in resting muscle. During exercise, this is reversed and active muscle blood flow may be as high as 8 ml/min/g (Guyton and Hall, 2011). Vigorous exercise (60% of maximal aerobic capacity, $\dot{V}O_{2max}$) results in a reduction of RPF of almost one-quarter of baseline values (figure 1.1; Kenney and Ho, 1995). While during more vigorous activity (> 80% $\dot{V}O_{2max}$), falls in RPF can approach 50% from baseline (Walker *et al.*, 1994). Thus, reduction in RBF appears to be *intensity-dependent* with higher activity levels resulting in more profound reductions. Considering that there is up to a 400% increase in cardiac output during exercise, such declines suggest potent (and multiple) mechanisms must be at play to limit RBF.

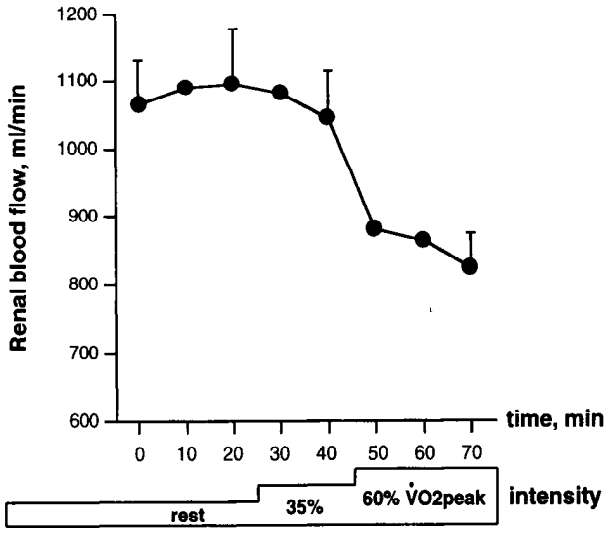

Figure 1.1. Renal blood flow as measured by p-aminohippuric acid clearance in young males (26 ± 2 yrs; solid circles; n = 6) at rest and during exercise at two different intensities. Values of the subjects fell by 24 ± 2% from baseline when 60% of $\dot{V}O_{2\ peak}$ was encountered demonstrating the profound effect of exercise intensity on renal blood flow. Values are means ± SE. Adopted from Kenney and Ho, 1995. $\dot{V}O_{2\ peak}$, peak aerobic capacity (highest $\dot{V}O_2$ observed).

Conversely, the *duration* of activity appears to play a less prominent role: prolongation of a light exercise load from 45 to 90 minutes does not impact upon effective RPF (Castenfors 1967a). Similarly, during prolonged heavy exercise there is a further, albeit small, reduction in RBF that does not have discernible impact upon maintaining haemodynamic stability. The reductions in RBF cease after exercise as evidenced by urinary clearance of p-aminohippuric acid returning to almost baseline levels within 60 minutes of ceasing both light and heavy intensity exercise (Grimby 1965b; Castenfors 1967a).

Potent neurohormonal influences are responsible for the reductions in RBF during exercise. The increased sympathetic drive during exercise promotes efferent and afferent arteriolar vasoconstriction, leading to increased vascular resistance (Tidgren *et al.*, 1991). Specifically, this is through release of noradrenaline and activation of the renin-aldosterone-angiotensin axis. Beta-adrenergic stimulation of the juxtaglomerular cells lead to the release of renin, which is augmented during exercise once a sufficient intensity has been attained.

Despite the powerful renal vasoconstrictive effects of noradrenaline and angiotensin, these are partly counterbalanced by the renal synthesis of prostaglandins such as prostaglandin E_2, which vasodilates afferent arterioles in the aim of maintaining adequate renal perfusion (Therland *et al.*, 2004). This is of particular importance for the continued functioning of deep medullary nephrons that survive at critical partial pressures of oxygen of no more than 10 kPa (Leonhardt and Landes, 1963). Cyclooxygenase-2 inhibitors such as non-steroidal anti-inflammatory drugs prevent prostaglandin synthesis and over-the-counter preparations such as ibuprofen are commonly used amongst athletes as an analgesic to treat minor musculoskeletal ailments (Warden, 2010). This may shift the balance of maintaining RBF during exercise towards vasoconstriction and contribute to further physiological stress and kidney dysfunction (figure 1.2; Walker *et al.*, 1994; Sanders, 1995).

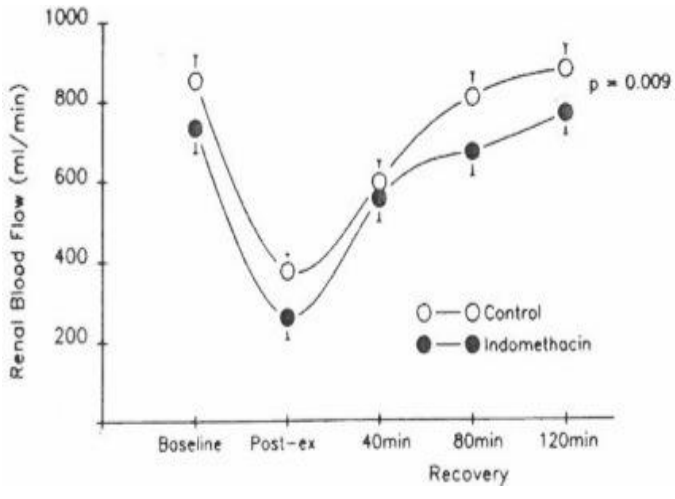

Figure 1.2. Renal blood flow (as measured by p-aminohippuric acid) in eight healthy males who performed treadmill exercise for 30 min at 80% maximal aerobic capacity. Following control bout (open circles), the group repeated exercise one week later having taken 250mg of the non-steroidal anti inflammatory (indomethacin) 36hr previously (filled circles). Indomethacin produced a greater reduction in renal blood flow post-exercise compared to control (P = 0.009). Adopted from Walker *et al.*, 1994.

1.1.2. *Glomerular filtration rate.* Glomerular filtration rate (GFR) is defined as the volume of fluid filtered from the glomerulus into Bowman's capsule per unit time (Guyton and Hall, 2011). Similar to RBF, determining GFR ideally requires a solute that is freely filtered and is neither secreted nor reabsorbed by the nephrons. Thus, with this principle in mind, the following equation can be derived for such a solute if its urine (U_x), plasma (P) concentrations and urine flow rate (V) are known [1]:

$$GFR = \frac{U_x . V}{P}$$

Inulin, a naturally occurring fructan polysaccharide, is filtered by glomeruli but does not undergo tubular secretion or reabsorption, and therefore can be used to determine GFR by the above method (Erd *et al.*, 1969). In humans, this can be administered as a continuous intravenous infusion with measurements of plasma and urine concentrations and urinary flow rates taken at chosen time intervals. Iohexol, a contrast medium, can be utilised in a similar fashion but a single injected dose followed by several measurements of plasma clearance over time is required (Krutzén *et al.*, 1984).

Use of inulin is considered a "gold standard" for GFR estimation and was applied in many early studies of kidney physiology during exercise. However, this method does present practical challenges, including insertion of intravenous and urinary catheters that may be uncomfortable for subjects during strenuous physical activity, and rare hypersensitivity reactions to intravenous inulin infusions (Bacchetta *et al.*, 2008).

A less invasive and labourious method involves determining a urinary creatinine clearance (Hayman *et al.*, 1933). Creatinine (Cr) derived from creatine phosphate found in muscle. Typically, over a 24-hour period, urine is collected to determine the amount of Cr excreted. Similar to above, the Cr concentrations in urine (U_{Cr}) and a single recording from plasma (P_{Cr}) together with a urine flow rate (V) can be used to calculate a urinary Cr clearance (Cr_{cl}):

$$Cr_{cl} = \frac{U_{Cr} . V}{P_{Cr}}$$

[1] The GFR calculated is assumed to be the GFR for all nephrons within the kidneys.

Apart from the practical issues surrounding urine collection other drawbacks of urinary Cr clearance lie in the numerous factors that may influence Cr metabolism, including: age, sex, ethnicity and muscle mass (Stevens and Levey, 2005). Also, in the specific context of exercise, use of creatinine may be flawed (see below) and as the test is typically performed over a 24-hour period, this makes it less suitable for the study of the acute effects of exercise upon renal function.

Kidney physiology studies in the last decade have moved away from such invasive and laborious measurements to using serum or plasma Cr concentrations-based equations that generate an estimated GFR to reflect acute changes in kidney function during exercise (Banfi *et al.*, 2009). However, the application of estimated GFR here is questionable. Aside from the issues inherent in using Cr as a marker of kidney function, GFR-based equations have been derived from specific patient populations with stable CKD and not from healthy exercising populations (Levey *et al.*, 1999). Also, the equations assume stable Cr metabolism (i.e. relatively constant rates of production and excretion), however, this is not the case when there is abrupt impairment in kidney function (e.g. an acute kidney injury), or during intense exercise when muscle breakdown leads to increases in blood Cr. Indeed, if blood Cr is measured too early, falsely low or normal readings could result as the time required for a new steady state of Cr metabolism can exceed 24 hours (Chiou and Hsu, 1975). Hence, GFR would be under-estimated in such circumstances (Hostetter *et al.*, 2010).

Despite these limitations, the ease of obtaining this approximation, its low cost and widespread use in many clinical and research settings has made it popular amongst exercise physiologists investigating kidney function. Most studies utilizing this method have shown that GFR is unchanged during light to moderate exercise (Grimby 1965b, Poortmans *et al.*, 2013). However, with increasing intensity (measured by % maximal heart rate or % $\dot{V}O_{2max}$), there is a systematic decrease in GFR where up to a 30% reduction from baseline may be seen (figure 1.3; Poortmans and Vanderstraeten, 1994, Poortmans *et al.*, 2013). The decrease in GFR appears to be maximal immediately following exercise and extends beyond 60 minutes post-exercise during endurance based-events. A return of GFR back towards baseline levels may take several hours (Neumayr *et al.*, 2003; Lippi *et al.*, 2008, Mingels *et al.*, 2009).

As with control of RBF during exercise, adjustment of GFR is dependent upon the relative vascular tone between the efferent and afferent arterioles. Accordingly, it follows that a reduction of RBF during exercise leads to a similar effect on GFR and that this phenomena is again intensity-dependent. However, there are some distinct differences between GFR and RBF. Post-exercise, GFR decreases relatively less than RBF. This latter observation is

important because if the kidneys do not excrete the accumulated nitrogenous waste products from protein catabolism during exercise, then a toxic uraemia may potentially develop (Haralambie and Berg, 1976; Priest *et al.*, 1982, Lemon *et al.*, 1989).

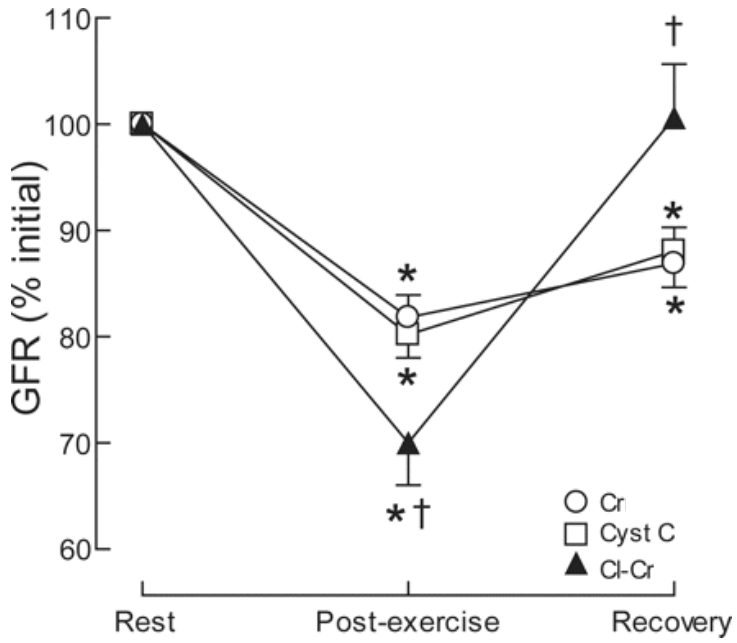

Figure 1.3. Changes (expressed as % rest) in glomerular filtration rate (GFR) following 30 minutes of treadmill exercise at 80% maximal aerobic capacity as estimated by serum creatinine (Cr; open circles), cystatin C (Cyst C; open squares), and by Cr clearance (Cl-Cr; filled triangles). Exercise resulted in all three measures indicating a marked reduction in GFR post-exercise, but more so for Cl-Cr (-30.0%) compared to Cyst C (-19.8%) and Cr (-18.2%). Values are means ± SD. GFR, glomerular filtration rate. * Denotes significant differences with rest values ($P < 0.05$). † Denotes significant differences with Cr and Cyst C ($P < 0.05$). Adopted from Poortmans *et al.*, 2013.

An important concept related to GFR and RBF is the filtration fraction. This represents the proportion of fluid within glomeruli which passes through into the tubules. Mathematically, it can be represented as follows, where RPF is considered as a surrogate for RBF:

$$Filtration\ Fraction = \frac{GFR}{RPF}$$

The resting filtration fraction is 0.2 / 20%. Due to the relative preservation of GFR to RPF, filtration fraction increases during exercise in an intensity-related fashion. Castenfors (1976b) demonstrated that a short severe bout of exercise could result in a filtration fraction of 0.45.

Although good experimental evidence is lacking, there are some plausible explanations including a relatively greater increase in efferent compared to afferent vascular tone (thus increasing intraglomerular pressures), and sluggish transit through glomeruli permitting more time for passage of fluid and other metabolites into the tubular space (Hohimer and Smith, 1979). This latter mechanism may have a significant role to play in the manifestation of post-exercise proteinuria and haematuria.

1.1.3. *Urine production.* Generally, urine production and flow falls during and after exercise (Poortmans 1984, Bellingheri *et al.*, 2008). Indeed, this is one of the few post-exercise observations that is apparent to the subject (the other being visible haematuria), but seems physiologically appropriate, as the kidneys need to maintain intravascular volume and therefore blood pressure during heavy physical exertion.

However, the magnitude, duration and rate of decrease of urinary flow are not readily predictable. Indeed, Kachadorian and Johnson (1970) found that low levels of exercise were associated with increases in urine flow and sodium excretion. However, once higher exercise intensities are performed there is a decrease in free-water clearance, and even hyperhydration prior to or during severe exercise does not prevent the decrease in urine flow post-exercise (Refsum and Strömme, 1975; Virvidakis *et al*., 1986). Anti-diuretic hormone (ADH) plays a significant role in these observations as a fall in plasma concentrations permits higher urinary flow rates during low intensity exercise, whereas concentrations can increase almost three-fold during higher intensity activity (Freund *et al*., 1991). Furthermore, ADH release can be increased by emotional components such as pain, high fluid losses, high ambient temperature and other hormonal factors such as renin, aldosterone and atrial natriuretic peptide (ANP, Cuneo *et al*., 1987; Galperin *et al.*, 2006).

A strong positive correlation has been observed between urinary flow rates and GFR at low exercise intensities (e.g. ~25% $\dot{V}O_{2max}$; Freund *et al*., 1991). Apart from low plasma ADH at such workloads, concomitant increases in plasma ANP have been suggested as a contributory factor (Sosa *et al.*, 1986). Similar to above, once sufficiently high intensities of exercise are achieved (≥80% $\dot{V}O_{2max}$), GFR, urine flow and urine osmolality actually decrease, counteracting the effects of ANP (Freund *et al.*, 1988). Increased sympathetic activity and falls in RBF through afferent vasoconstriction are again implicated, but the excretion of dilute urine suggests that concentration mechanisms are also being impaired (figure 1.4.; Wade, 1984).

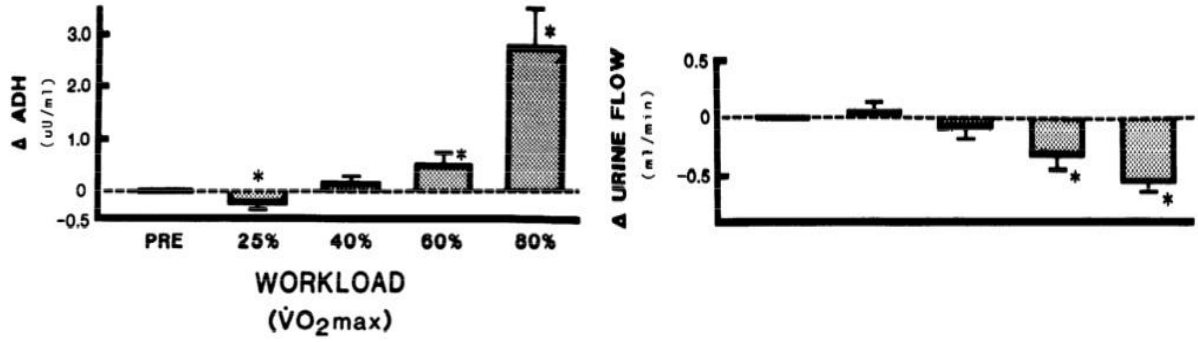

Figure 1.4. Changes in plasma anti-diuretic hormone (left panel) concentration and urine flow rates (right panel) in eight healthy male subjects who underwent a graded cycle ergometry at different workloads (25, 40, 60 and 80% $\dot{V}O_{2max}$). As workload increased, urine flow rates fell and there was a concomitant increase in plasma anti-diuretic hormone concentrations which was particularly marked between 60 to 80% $\dot{V}O_{2max}$. Values are means ± SD. ADH, anti-diuretic hormone; * Denotes significant differences with pre values ($p<0.05$). Adopted from Freund *et al*., 1991.

1.1.4. *Post-exercise proteinuria.* The presence of protein in human urine was first documented by Morner in 1895 who commented upon a "*mucodahliche Substantz*" in military recruits (Morner, 1895). It is now recognised that protein excretion in the urine of healthy resting subjects may be up to 150 mg / day (Franklin, 1959). The vast majority of this consists of Tamm-Horsfall mucoprotein produced by the thick ascending limb of the loop of Henle (Tamm and Horsfall, 1950; Pennica *et al.*, 1987). This can present either as a monomer of approximately 68 kDa or more commonly as large aggregates of several million Da. Globulin fractions account for 5-10% of total urinary protein of which approximately 50% are not found in the plasma or are only detectable in trace amounts (Poortmans and Jeanloz, 1968, Poortmans and Vanderstraeten, 1994). These glycoproteins have a molecular weightof 10-40 kDa and include amongst others: immunoglobulin kappa and lambda chains, β_2-microglobulin and lysozyme.

From a practical perspective, initial rapid detection normally involves use of urine dipsticks ("multistix") that give an approximate quantitative indication of total proteinuria but are prone to false-negative and false-positive results (Bonnardeaux *et al.*, 1994). Many early physiological studies expressed post-exercise proteinuria as an excretion rate in µg/min to account for a concentration effect. However, recent studies have moved to using urine protein-to-creatinine or albumin-to-creatinine ratio (P:Cr or A:Cr, respectively), which has superseded the need for 24-hour proteinuria collections in the clinical setting with only a spot-urine sample required (figure 1.5; Poortmans and Vanderstraeten, 1994; Siu *et al.*, 2011).

Regardless of widespread use, it is yet to be clarified whether this is a valid measure given the instability of creatinine handling by the kidneys during exercise as discussed above.

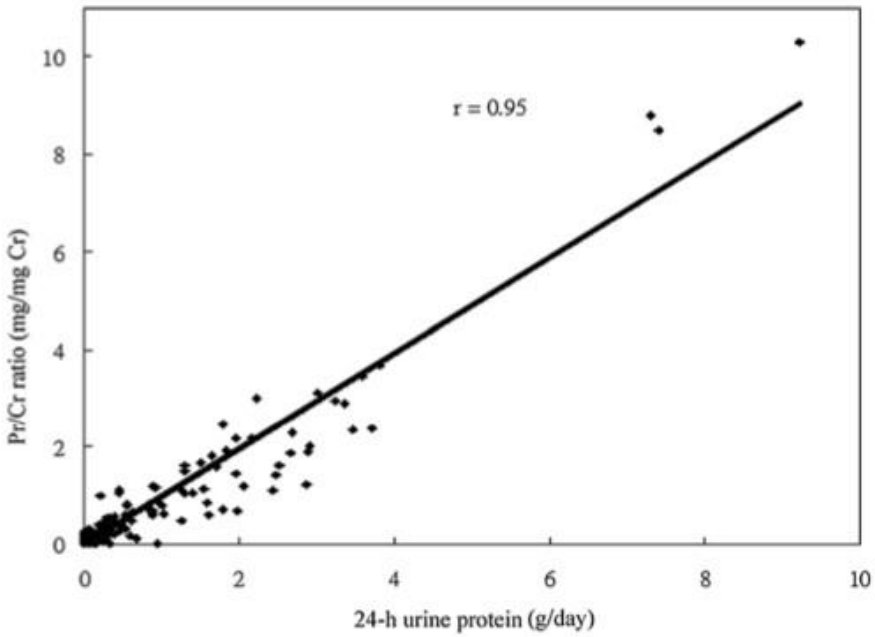

Figure 1.5. There a strong positive correlation (r = 0.95; $P < 0.0001$) between spot urine protein-to-creatinine (Pr/Cr) ratio and concurrent 24-hour urine protein excretion as demonstrated in 174 adult patients with chronic kidney disease. Similar findings have been noted in many other studies and consequently use of protein-to-creatnine ratio has supplanted the use of 24-hour proteinuria estimations in routine clinical and research settings. Adopted from Siu *et al.,* 2011.

Post-exercise proteinuria is one of the more thoroughly investigated exercise-induced renal responses (Poortmans 1977, Bellingheri *et al.*, 2008). Young individuals without any evidence of renal disease can increase resting urinary albumin excretion rates as high as 100-fold following intense exercise (Poortmans *et al.*, 1981; Poortmans 1985, Poortmans and Vanderstraeten, 1994). It does not appear to have a precise quantitative definition, but recent studies that utilised A:Cr have cited a rise of more than 3 mmol/mg from resting values i.e. within the range of pathologic proteinuria at rest (Heathcote *et al.*, 2009). Previous observational studies revealed a variable prevalence depending on the physical discipline: boxers (25%), wrestlers (100%), gymnasts (13-55%), footballers (80%) and oarsmen (100%), but factors such as intensity and duration of exercise were often uncontrolled (Bellinghieri *et al.*, 2008). Regardless, runners consistently appear to excrete more protein than cyclists, swimmers and rowers despite having similarily high plasma lactate levels after exercise (Poortmans *et al.*, 1982). Moreover, this phenomenon is not observed in all participants, even with strenuous exercise and individuals who already have indications of significant proteinuria at rest (e.g. nephrotic-range proteinuria - greater than 3g / 24 hours; Cruz *et al.*,

1989). Constitutional factors may be relevant as protein excretion for exhaustive bicycle ergometry is highly correlated in monozygotic but not dizygotic twins (Liljefors *et al.*, 1969).

As with other physiological phenomenon during exercise, post-exercise proteinuria is driven by exercise intensity (Poortmans, 1984). This is supported by increased proteinuria rates following sprints compared to longer endurance-based events (figure 1.6).

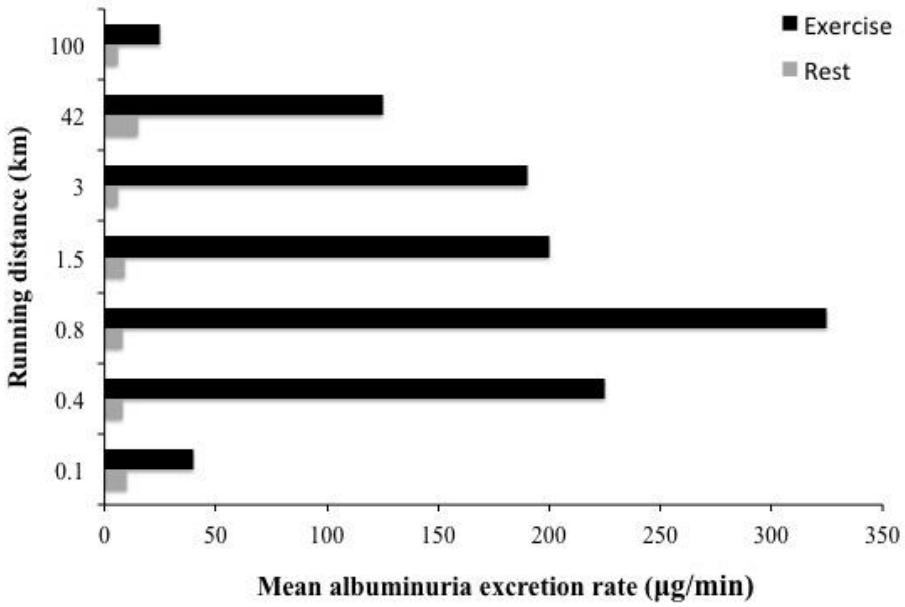

Figure 1.6. Mean albuminuria excretion rates at different absolute intensities of exercise as measured by running distance. Grey bars represent mean values at resting state. Black bars are mean values following exercise. A 800 metre sprint has the highest propensity for post-exercise proteinuria. This progressively falls as longer distances are encountered. However, very short-term exercise (e.g. 100 metre sprint) has a lesser effect than longer runs. Adapted from Poortmans, 1994.

The pattern of protein excreted in post-exercise urine differs to the normal resting condition (Poortmans and Jeanloz, 1968). Through urine electrophoresis of post-exercise urine, an increase in glomerular permeability is evidenced by the presence of albumin and other larger macromolecules such as immunoglobulin G, α_1-acid glycoprotein and α_1-antitrypsin (figure 1.7; Poortmans and Lion, 1963; Poortmans and Jeanloz, 1968). Moreover, more plasma protein seems to pass through into the urine after exercise than at rest and this accounts for over 80% of total protein excretion after exercise (Poortmans and Jeanloz, 1968). For diseased states, the type of proteinuria encountered can vary according to aetiology. For example, in multiple myeloma (a disease characterised by an abnormal proliferation of B-lymphocytes), excess synthesis of abnormal monoclonal immunoglobulins can spill-over into the urine and result in an overflow proteinuria. Conversely, in disease states where there is an excess loss of normal proteins from plasma into the urine (e.g. nephrotic syndromes),

proteinuria can be highly selective as demonstrated by a high urinary IgG:transferrin ratio. However, regardless of the aetiology, excessive and persistent albuminuria is indicative of CKD (Heathcote *et al.*, 2009).

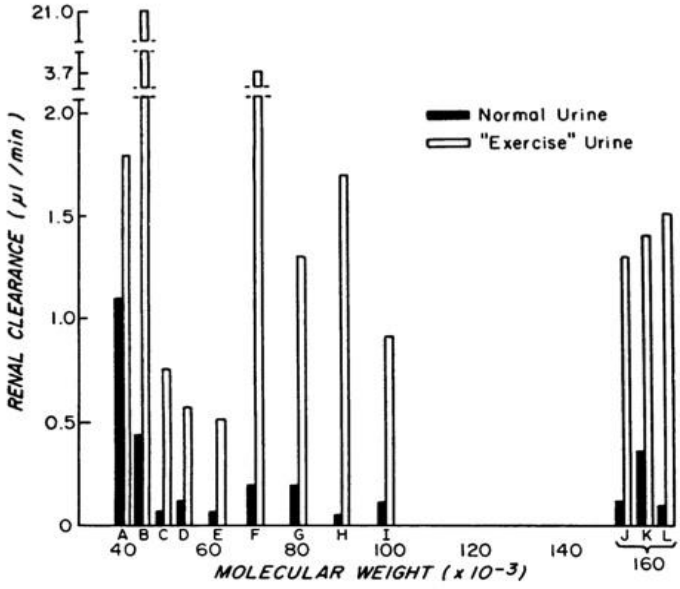

Figure 1.7. Comparison between the molecular weight of proteins and their renal clearance under normal resting conditions (filled bars) and post-exercise (unfilled bars). The letter under each pair of columns indicates the plasma protein studied: A, β_2-glycoprotein I; B, α_1-acid glycoprotein; C. α_1-antitrypsin D, Gc-globulin; E, tryptophan-rich prealbumin; F, albumin; G, hemopexin; H, transferrin; I, haptoglobin; J, cerulplasmin; K, γA-globulin; and L, γG-globulin. It is clear that proteins of a higher molecular weight are excreted following exercise suggesting an increase in glomerular permeability. Adopted from Poortmans and Jeanloz, 1968.

Several postulated mechanisms have been put forward to explain post-exercise proteinuria. Firstly, an acidic environment during exercise as evidenced by lactate elevations is thought to affect glomerular permeability (Poortmans and Labilloy, 1981). Suzuki and Ikawa (1991) demonstrated that increased lactate production following strenuous exercise resulted in excretion of both albumin and low-molecular weight proteins, but also observed that increased organic acids could change glomerular permeability by decreasing surface anionic charge and inhibiting tubular reabsorption. In animal studies, intravenous injection of hydrochloric acid with subsequent metabolic acidosis induced proteinuria in rodents (Poortmans 1977). Moreover, this observation is supported by studies in humans that have shown a decrease in exercise-induced proteinuria following infusion of sodium bicarbonate to correct lactic acidosis (Bellinghieri *et al.*, 2008).

Secondly, as discussed in the preceding section, the vasoconstriction of renal arterioles during exercise induces a more marked reduction of RBF compared to GFR and therefore increased filtration fraction (Castenfors 1967b). Indeed, inducing vasoconstriction experimentally though an infusion of noradrenaline in man at rest yields an approximately 20-fold increase in proteinuria (Cantone and Cerretelli, 1960; Taylor 1960). The increased hydrostatic pressures that results (due to a relatively greater efferent to afferent arteriolar vasoconstriction), coupled with sluggish kidney blood flow is likely to enhance diffusion of macromolecules into the tubular lumen (Hohimer and Smith, 1979).

Thirdly, upregulation of renin during exercise through heightened sympathetic activity and stimulation of the juxtaglomerular apparatus may also be influential, as proteinuria can be induced experimentally by intravenous renin injection (Pickering and Prinzmetal, 1940; Deodhar *et al.*, 1962). Kallikrein, an enzyme of the kinin system, could also be involved, as proteinuria is provoked when kallikrein is injected into rabbits and several kinin-releasing enzymes are found in urine after healthy subjects perform proteinuria-inducing exercise (Murakami *et al.*, 1968; Masamura *et al.*, 1970).

The final theory states that exercise proteinuria results from a loss of fixed negative charges on the capillary wall that leads to a loss of charge-dependent glomerular permselectivity (Deen and Satvat, 1981). This has been shown experimentally by infusing the polycation hexadimethrine that neutralises the polyanions of the glomerular basement membrane in rats and induces an acute reversible proteinuria (Hunsicker *et al.*, 1981; Bridges Jr *et al.*, 1991). However, this experimental glomerular injury is only observed in rats, as in exercising humans immunoelectrophoresis does not reveal any differences between the fixed charges of proteins in resting and post-exercise urines (Poortmans 1984).

When higher intensity activity is encountered, tubular proteinuria becomes more prominent (up to 100-fold from baseline) with the appearance of lower molecular weight proteins (< 40 kDa) such as lysozyme and β_2-microglobulin. This suggests impairment of tubular reabsorption due to saturation of reabsorptive mechanisms (Miyai and Ogata, 1990). Nevertheless, albumin still constitutes the majority of protein excreted. Thus, post-exercise proteinuria following severe exercise is of the mixed glomerular-tubular type (Poortmans *et al.*, 1988; Poortmans and Vanderstraeten, 1994).

Maximal proteinuria occurs 20-30 minutes following exercise (Poortmans and Vanderstraeten, 1994). Both urinary total and albumin excretion rates follow a logarithmic decline with a half-life of about one hour and resting levels achieved 24-48 hours after activity (Coye and Rosandich, 1960). Persistence beyond this time period should raise the

need for further investigation (Bellinghieri *et al.*, 2008). To date, it has been assumed that post-exercise proteinuria is not associated with any long-term sequalae such as CKD. The evidence for this is almost non-existent and is partly derived from the benign nature of other forms of physiological proteinuria e.g. orthostatic proteinuria (Springberg *et al.*, 1982). This is a tenuous association as individuals with post-exercise proteinuria may experience greater degrees of renal stress compared to those without (Bellinghieri *et al.*, 2008).

1.1.5. *Post-exercise haematuria.* The first description concerning the presence of blood in the urine may have been over 2000 years ago (Fine 1986). The incidence of haematuria in normal resting subjects varies between 13-38% depending on the population studied and the definition used as haematuria can denote the presence of erythrocytes *per se* or apparent haematuria such as myoglobinuria or haemoglobinuria (Rockall *et al.*, 1996). Haematuria is properly defined as *microscopic* when greater than three erythrocytes per high power field or when 1000 erythrocytes / ml urine is noted (Froom *et al.*, 1984; Mohr *et al.*, 1986; Rockall *et al.*, 1996). Haematuria may also present *macroscopically*, where blood and / or clots can be visible to the subject. The latter invariably leads to distress and often leads to physicians conducting further investigations.

Phase-contrast microscopy can be used to determine their origin from the renal tract: dysmorphic erythrocytes transit through glomeruli and tubules leading to deformity of their membrane, whereas erythrocytes of a normal appearance are likely to have arisen post-glomeruli e.g. from the bladder (figure 1.8; Pillsworth Jr *et al.*, 1987).

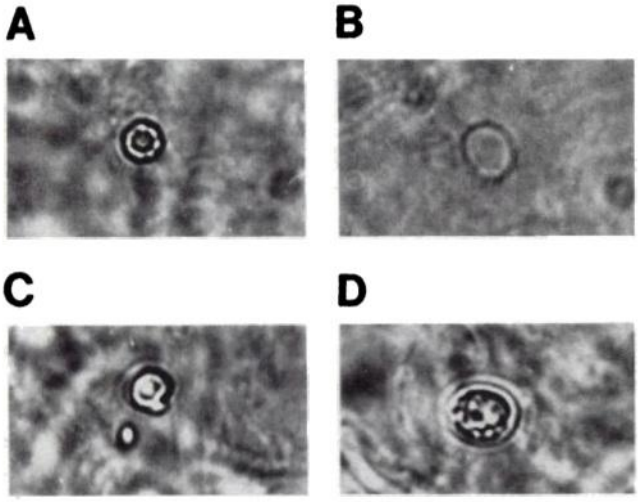

Figure 1.8. Examples of normal and dysmorphic erythrocytes as seen by phase-contrast microscopy (at x400 magnification): A) normal erythrocyte; B) ghost erythrocyte (where haemoglobin has leached out); C) dysmorphic erythrocyte extruding small bleb from membrane; D) dysmorphic erythrocyte with ruptured membrane. Adopted from Pillsworth Jr *et al.*, 1987.

Like proteinuria, initial detection commonly involves the use of a urine multistix that are readily available and allow rapid detection but may not be able to differentiate reliably between true and apparent (i.e. false-positive) haematuria (Doezema and Standefer, 1990). For haematuria, the urine multistix utilises a pseudoperoxidase in haemoglobin that catalyses a reaction between hydrogen peroxide and tetramethylbenzidine resulting in a green oxidised chromogen (Martin *et al.*, 1984). Free haemoglobin produces a colour change from yellow (negative) to blue (strongly positive). In contrast, when whole erythrocytes make contact with the test strip, the liberated haemoglobin results in a speckled pattern with the the detection of haematuria. With this method, most studies have demonstrated 91-100% sensitivity, while the specificity is more variable at 65-99% (Gleeson *et al.*, 1995; Worrall 2009). False positive and negative results may occur as the reagents react with a host of other substances and accounts for the variable specificity e.g. vegetable peroxide, *Escherichia Coli* and myoglobin (Gibson and Le Seve, 1986). Thus, any suspicion of true haematuria should be evaluated formally by a wet-slide examination from fresh urine (Schumann and Schumann, 1992).

Ramazzini described haematuria following exercise in 1713 after studying the urine of runners, but the first formal description of exercise-induced hematuria is credited to Barach in 1910, who detected erythrocytes through microscopy in the urine of 18 marathon runners after a 40-kilometre race (Barach 1910; Brieger 1980; Franco 1999). Postulated mechanisms for exercise-induced haematuria vary and are both intensity and duration-based (Jones and Newhouse, 1997). This can be partly explained through the type of physical activity where potentially different stresses are placed upon erythrocytes and the kidneys.

With reference to intensity–based mechanisms, the reduction in RBF together with vasoconstricting effects promotes a relative hypoxia within the kidney (Helzer-Julin *et al.*, 1988). This may lead to increased fragility of spiral vessels that connect interlobular arteries to the capillary bed and following exercise their contents may leak into minor calyces for excretion when resting renal blood supply resumes (Baker, 1959). Catechoamines themselves may promote erythrocyte destruction by two means: i) through release of a haemolysing factor (lysolecithin) from splenic contraction, and ii) via a direct effect on erythrocytes which increases osmotic and mechanical susceptibility (Newhouse and Clement, 1988; Szygula, 1990).

Historically, the most commonly documented cause of exercise-induced haematuria is the footstrike haemolysis (march haemaglobinuria) that is noted in long-distance runners due to physical trauma of erythrocytes circulating close to the sole of the foot (Horder and Horder, 1970; Dufaux *et al.*, 1981; Eichner, 1985). The haemoglobin released from this form of

haemolysis usually complexes with plasma haptoglobin. However, when this mechanism becomes saturated then overspill into the urine occurs (Jones and Newhouse, 1997). This mechanism is further supported by haemolysis being greater in running compared to swimming, downhill running versus uphill running, and running in hard-soled relative to running in soft-soled shoes (figure 1.9; Buckle, 1965; Miller *et al.*, 1988). Other potential mechanisms affecting erythrocyte membranes during exercise include peroxidation due to free radical production during exhaustive exercise, dehydration affecting erythrocyte osmolarity, increased circulation rate, and elevated core body temperature (Clement and Sawchuck 1984; Szygula, 1990; Sjodin *et al.*, 1990).

Potentially independent of intensity and duration, bladder and kidney trauma is another plausible mechanism by which haematuria can arise e.g. jostling of the bladder and kidneys during running. Cystoscopic examination in runners 48-hours post-exercise has demonstrated bladder wall contusions which can predispose to haematuria (Blacklock, 1977). Furthermore, direct trauma to the kidneys during traumatic sports such as boxing can yield haematuria. Aside from physical force, a crouched body positioning and a lack of cushioning perirenal fat may also be contributory (Kleiman, 1958).

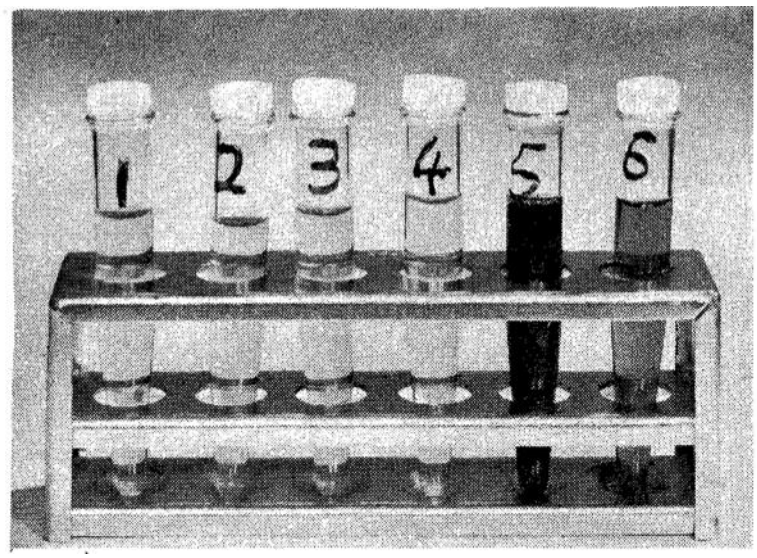

Figure 1.9. Urine samples taken during a seven-mile run in an individual prone to march haemoglobinuria. Tubes 1, 2 and 3 represent urine taken before, 20 and 60 minutes after running with insoles, respectively. Tubes 4, 5 and 6 represent the same time points of urine sampling, but taken whilst running without insoles. With the insoles there was no detectable change in plasma haemoglobin or haptoglobin. Adopted from Buckle, 1965.

As discussed in section 1.1.1, non-steroidal anti-inflammatory medications are used often by athletes and can affect RBF adversely through prostaglandin inhibition and exacerbate any exercise-related reductions in RBF. As the renal papillae within the medullary region are especially dependent upon prostaglandin E_2-dependent blood flow, this mechanism can partake in the development of a renal papillary necrosis with sloughing of papillae that presents with macroscopic haematuria, urinary obstruction and loin pain (Henrich 1998).

1.1.6. *Urinary sediments*. Apart from erythrocytes, a variety of other cellular elements are found in post-exercise urine including white blood cells (leukocytes), epithelial cells, and casts. Under resting conditions, three leukocytes and epithelial cells of a normal population may be seen (Geyer 1993). Casts are usually hyaline and comprises of Tamm-Horsfall protein that originates from the dropout of distal convoluted tubular cells (Tamm and Horsfall, 1950; Pennica *et al.*, 1987). Exercise leads to marked increases in other sediment elements: Poortmans (1977) found a mean urinary excretion of 280 erythrocytes, 900 leucocytes and 365 epithelial cells per minute after strenuous exercise in 90 healthy young men. Most of the casts were hyaline.

The presence of granular casts may also occur after heavy exercise and is associated with tubular damage and an acute disruption in kidney function (Geyer 1993). Howenstein (1960) performed renal biopsies in three marine recruits following intensive squat jumping exercise and reported adhesions between the Bowman's capsule and the glomerular tuft, pigmented debris in the tubular lumens and dilated tubules whose lumens contained a granular cast.

1.1.7. *Electrolyte metabolism.* Heavy exercise appears to generally inhibit electrolyte excretion – especially that of sodium (Grimby 1965a; Castenfors 1967a; Kachadorian 1972). This makes physiological sense if the exercising body is to maintain intravascular volume and blood pressure. Rather than being due to declines in GFR, increased tubular reabsorption is the major influence for reduced sodium loss. Sympathetic stimulation leading to activation of the renin-angiotensin-aldosterone axis and increased levels of aldosterone, promotes both sodium and water reabsorption from the collecting duct (Freund *et al.*, 1991). The rise in plasma aldosterone activity is related to both intensity and duration of exercise and there is an inverse relationship between plasma aldosterone and urine output (Galbo 1982). Other electrolytes such as chloride, calcium and phosphorus also demonstrate similar reductions in excretion after exercise, although potassium excretion, in contrast to the other electrolytes, may vary (Poortmans, 1984).

With respect of urine osmolality, short-term exercise of moderate intensity is associated with an increase in urine osmolality of about 40 mOsm/l. However, as intensity of exercise increases, osmolality decreases implying a defect in tubular concentrating mechanisms.

Generally, most urinary electrolyte changes during exercise do not have clinical consequences. However, the combination of exercise-related increases in aldosterone, and ADH, and regular fluid intake (including hyperhydration) during prolonged endurance activity, can lead to a progressive dilution of plasma and its sodium content (O'Connor 2006; Draper *et al.*, 2009). Biochemically, this manifests as hyponatraemia with a low plasma sodium concentration, low plasma osmolality and comparatively higher urine osmolality. Exercise-induced hyponatraemia is potentially life-threatening when developed acutely. Non-specific symptoms such as lethargy, nausea, vomiting can progress to reduced consciousness levels and fitting owing to the development of cerebral oedema; particularly when lower plasma sodium concentrations of less than 115 mmol/l are encountered (O'Connor 2006). Treatment involves a careful assessment of fluid balance upon presentation and in most cases a fluid restriction is sufficient. In instances of repeated fitting and reduced consciousness, ventilator support, benzodiazepines and hypertonic saline may be indicated (Hew-Butler *et al.*, 2008).

1.2. *EXAMINING THE BOUNDARY BETWEEN PHYSIOLOGICAL STRESS AND PATHOLOGICAL STATES DURING STRENUOUS PHYSICAL EXERTION*.

The preceding discussion has outlined the changes to kidney physiology during exercise in healthy humans and so far could be regarded as normal phenomena. Nonetheless, they clearly exhibit a potential to crossover into a pathological condition. For example, reductions in RBF, GFR, proteinuria and haematuria are all characteristic clinical features of acute and chronic kidney disease. If exercise-induced changes do not revert back to resting values shortly following activity, prolonged interruptions to RBF and GFR could manifest as nitrogenous waste accumulation, severe electrolyte disturbances, and hypervolaemic states – all of which are life-threatening. Likewise, chronic proteinuria can be damaging to the kidney in the long-term as it contributes to a pro-inflammatory state associated with fibrotic changes of the renal parenchyma (Abbate *et al.*, 2006).

1.2.1. *Endurance exercise and troponin elevation.* The notion of physiological stress resembling a pathological state during heavy physical exertion has been investigated extensively in the heart. Like the kidneys, prolonged changes in normal physiological responses during exercise such as elevated cardiac output, heart rate and systolic blood pressure in conjunction with the physiologic milieu of endurance activity (e.g. elevations in reactive oxygen species, altered pH, and increased core temperature), could hypothetically damage cardiomyocytes (Shave *et al.*, 2010). Interestingly, similar factors have also been found to influence acute and chronic cardiac diseases (Ono *et al.*, 2004)

The ever-growing number of participants in marathons and similar events has permitted investigation of this possibility largely through the measurement of troponin-I and T-myocardial contractile proteins, which are sensitive and specific serum biomarkers of myocardial injury primarily used for the diagnosis of myocardial infarction (Thygesen *et al.*, 2007). Here, troponin is elevated post-endurance exercise to concentrations comparable to an established myocardial infarction (figure 1.10; Shave *et al.*, 2005; Collinson *et al.*, 2006; Shave *et al.*, 2010).

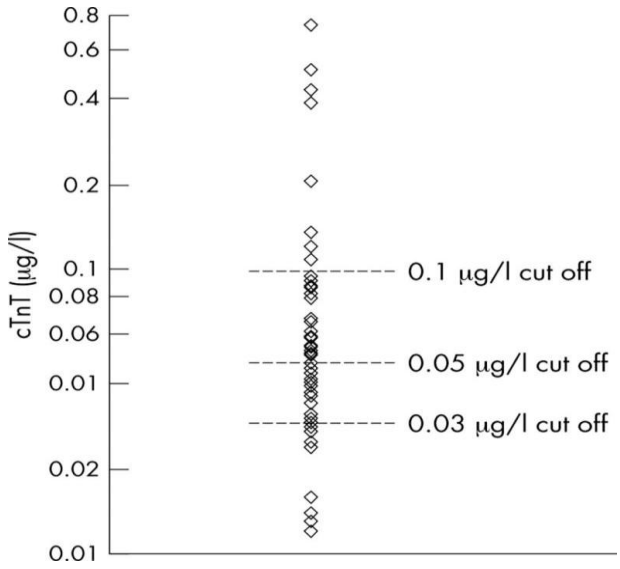

Figure 1.10. Cardiac troponin T measured in individual competitors following the London Marathon in 2005. Approximately 78% of runners demonstrated troponin T values above 0.01 µg/l, 58% above 0.03 µg/l, 36% above 0.05 µg/l and 11% above 0.1 µg/l. All cut-offs have been diagnostic of an acute myocardial infarction. cTnT, cardiac troponin T. Adopted from Shave *et al.*, 2005.

The reported incidence of this phenomena has ranged from 0 to 78% over the last two decades and this variability is partly due to the development of troponin-T immunoassays with improved sensitivity and specificity and application of different cut-off criteria (Shave *et al.*, 2005). Initial assays could not reliably discriminate between skeletal and cardiac muscle damage but the current 4th generation high-sensitivity troponin-T immunoassay possesses less than 0.001% cross-reactivity with skeletal troponin-T (cf. 12% cross-reactivity with 1st generation troponin-T assay) (Wu *et al.*, 1994b; Laslett *et al.*, 1996; Michielsen *et al.*, 2008).

Compared to the large number of observational studies in the literature, laboratory-based studies examining troponin release during controlled exercise are relatively few. Of those identified, some reveal modest elevations in troponin (0.02-0.04 µg/l) as early as 30 minutes into exercise (Middleton *et al.*, 2002). Similar to kidney-related phenomena, the highest elevations are encountered during exercise of the highest intensity (i.e. 100% ventilatory threshold) rather than prolonged duration (Fu *et al.*, 2009). Indeed, it has been shown that troponin-T elevations correlate inversely with training distance, suggesting that adaptation of the heart to strenuous exercise may decrease cardiac myocyte injury (Nelian *et al.*, 2006). This conclusion is supported by the similar kinetics of other biomarkers of cardiac stress such as proBNP (Shave *et al.*, 2007). Age and training status may also have some bearing on the degree of troponin rise encountered (Neumayr *et al.*, 2001; Fortescue *et al.*, 2007).

1.2.2. *Mechanisms of troponin release during endurance exercise.* The mechanism of troponin rise in endurance activity so far remains unclear. It could potentially reflect cardiac muscle stretch that results in cytosolic troponin leakage due to transient increases in myocyte

membrane permeability from the stress-induced overload of free radicals, hypoxia or ischaemia (McNeil and Khakee, 1992; Koller and Schobersberger, 2009; Goette 2009). This purported mechanism has similarities with exertional rhabdomyolysis of skeletal muscle where leakage of specific proteins such as creatine kinase and myoglobin also occurs (Warren *et al.*, 2002). Although this process is distinct from myocardial infarction where actual necrosis of the contractile apparatus occurs (Wu *et al.*, 1994b).

Another factor potentially contributing to troponin elevations is the decrease in GFR during exercise that may impact upon troponin excretion (see section 1.1.2.). Minor troponin rises in patients occur in patients with end-stage renal disease i.e. GFR of typically < 10 ml/min/1.73 m^2 (Wallace *et al.*, 2006). However, in this specific clinical context such elevated concentrations portend an increased risk of a future acute cardiovascular event (Sommerer *et al.*, 2007).

1.2.3. *Endurance exercise and cardiac imaging.* Apart from blood-based biomarker sampling, concomitant measures of cardiac function through imaging suggests transient decreases in systolic function and diastolic filling with an overall immediate post-exercise reduction in the ejection fraction, systolic blood pressure/end systolic volume pressure and early-to-late diastolic filling ratio (figure 1.11; Mousavi *et al.*, 2009; Middleton *et al.*; 2006). Even in the long term, diastolic abnormalities of the left and right ventricle may persist up to one month despite normalisation of systolic abnormalities (Neilan *et al.*, 2006).

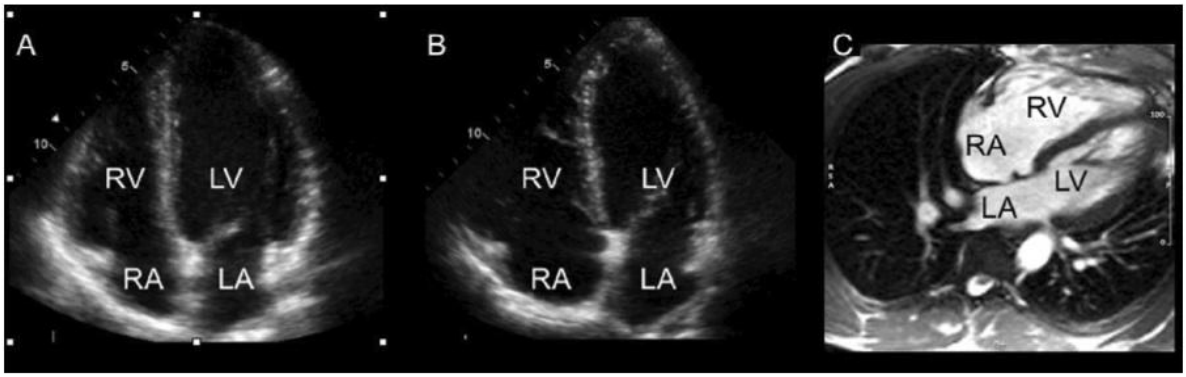

Figure 1.11. Transthoracic echocardiogram images in a marathon participant with a troponin-T concentration that increased from 0.01 to 0.54 μg/l post-marathon. *(A)* Baseline image demonstrating normal right ventricular structure and function. *(B)* Post-marathon evidence of mild right ventricular dilatation (right ventricular end-diastolic diameter 38 mm) and a decrease in right ventricular fractional area change of 35%. *(C)* Post-marathon cine-image in the horizontal long-axis view confirming a decrease in the right ventricular ejection fraction of 44%. LA: left atrium; LV: left ventricle; RA: right atrium; RV: right ventricle. Adopted from Mousavi *et al.*, 2009.

Nonetheless, the majority of non-invasive imaging data so far argue against an association of post-event troponin elevations and permanent myocardial injury (Siegel *et al.*, 1995; Shave *et al.*, 2010). In addition, the kinetics of troponin release following heavy endurance exercise bears little resemblance to that of an acute myocardial infarction and does not support irreversible cell death (Middleton *et al.*, 2008). The majority of troponin-T elevations post-exercise are short-lived and concentrations return back to normal within 24-48 hours after a strenuous event, whereas in myocardial infarction, troponin release follows a biphasic pattern with an initial release at two hours and a later release due to degeneration of the contractile apparatus (Shave *et al.*, 2010).

Probably the most convincing evidence of the benign nature of this occurence is that a large proportion of people who partake in regular strenuous exercise will have repeated elevations in cardiac biomarkers without coming to harm or clinical attention (Shave *et al.*, 2010). However, until the precise nature of cardiac biomarker up-regulation is resolved, physicians will still be surrounded by much confusion to whether this phenomenon is physiological or pathological. This situation is furthermore complicated by the widespread incidence of abnormal resting electrocardiograms in athletes (Wu *et al.*, 2006).

In contrast to the heart, the kidneys demonstrate specific instances whereby there is a clearer transgression to a pathological state from prolonged physiological stress during heavy physical exertion. The physiological stress may be the cause or contributory factor of an acute kidney injury that leads to a significant disruption of kidney function and might require urgent medical attention. Before describing these specific conditions in turn, it is necessary to move discussion into the clinical setting to define acute kidney injury and its relationship to kidney physiology during exercise.

1.3. *ACUTE KIDNEY INJURY*

Acute kidney injury (AKI), formally known as acute renal failure / insufficiency, is a clinical syndrome which can be simply defined as an abrupt or sudden insult to the kidneys with the end result being a reduction in kidney function (Webb and Dobb, 2007). The aetiology can be divided anatomically into three key areas: 1) pre-renal (disorders affecting perfusion to the kidneys); 2) renal (disorders affecting the kidney parenchyma); and 3) post-renal (disorders related to the structures post-kidney i.e. obstruction to ureters, bladder). The consequences of AKI can be life-threatening, particularly if dysfunction is prolonged and complications reflect an inability of the kidneys to maintain fluid, acid-base and electrolyte homeostasis e.g. hypervolaemic states, metabolic acidosis and hyperkalaemia (Mehta *et al.* 2007).

There is scarce data on the incidence of AKI and this has been marred by the previous lack of consensus on a working definition. However, hospital-acquired AKI appears to be more prevalent than the community form (7.1% vs. 1%; Kaufman *et al.*, 1991; Nash *et al.*, 2002). However, recent evidence suggests that community-acquired AKI, which can include AKI sustained from strenuous exercise, is at least as severe as hospital acquired AKI (Schissler *et al.*, 2013). The severity may result in the need for intensive care support and data from the UK-based Intensive Care National Audit Research Centre (ICNARC) suggests that AKI alone accounts for nearly ten percent of all intensive care unit bed days (Simpson *et al.*, 2005). Mortality in this setting can exceed 50%. Thus, AKI following heavy exertional activity is potentially a real public health concern, particularly when populations involved in strenuous physical activity are considered, e.g. amateur and professional sports players, or public services (e.g. military, firemen).

1.3.1. *Development of AKI definition.*

The term AKI represents a recent development in terminology and has arisen through the need to standardise an operative definition that can be used in both clinical and research environments (Mehta *et al.*, 2007). Previously, the definition varied according to the clinical environment; intensive care physicians, surgeons and emergency physicians all had unique definitions with differences based on their experience. This led to difficulties in determining the epidemiology and outcomes of AKI (Mehta *et al.*, 2007). The term AKI now denotes a spectrum of disease from mild kidney impairment to a more severe phenotype requiring supportive therapies such as dialysis (Bellomo *et al.*, 2004). Hence, a worldwide consortium was established to define stages of AKI that would indicate increasing severity of illness. The Acute Kidney Injury Network

(AKIN) criteria classifies AKI into three stages of progressive disease based upon elevations of serum / plasma Cr or decreased urine flow rates (table 1.1; Mehta *et al.*, 2007; Molitoris *et al.*, 2007). This has resulted in raising awareness of AKI amongst clinicians, allowing prognostication and facilitatation of a standardised approach to both audit and research.

AKI Stage	Serum creatinine (Cr) criteria	Urine output criteria
1	Increase ≥ 26 μmol/L within 48hrs or increase ≥ x 1.5 to x 1.9 reference Cr.	< 0.5 mL/kg/hr for > 6 consecutive hrs.
2	Increase ≥ x 2 to x 2.9 reference Cr.	< 0.5 mL/kg/hr for > 12 hrs.
3	increase ≥ x 3 reference SCr **or** increase ≥354 μmol/L **or** commenced on renal replacement therapy irrespective of stage.	< 0.3 mL/kg/hr for > 24 hrs or anuria for 12 hrs.

Table 1.1. Acute Kidney Injury Network criteria for acute kidney injury. Each stage indicates progressive severity of AKI. Either one of serum creatinine and urine output criteria has to be satisfied for each stage of AKI. It is envisaged that widespread introduction of this new system will enable healthcare professionals to consider the AKI as a spectrum of disease. Anuria, less than 100 mls urine / 24 h. Renal replacement therapy includes all forms of dialysis. Adapted from Mehta *et al.*, 2007.

1.3.2. *Traditional biomarkers of AKI.* Aside from Cr and urine output, there are other biomarkers that may be used to support or refine the diagnosis of an AKI. There is merit in performing smear examinations of a fresh urine samples to look for granular casts, as these are strongly suggestive of an acute tubular necrosis (Geyer 1993; Kanbay *et al.*, 2010). Calculation of a fractional excretion of sodium or urea helps to differentiate between pre-renal AKI and acute tubular necrosis (Schrier 2011). Also, ultrasonography of the renal tract at the earliest opportunity is recommended to ensure that urinary tract obstruction is excluded as a cause of AKI (Kalantarina, 2009a).

However, it should be noted that derangements of Cr, urine output are actually reflective of altered kidney function and not necessarily injury. Evidence for this comes from the observation that acute falls in GFR following kidney injury (i.e. as reflected by increased blood Cr), may take time to manifest until a new steady state in Cr metabolism becomes

established (Waikar and Bonventre, 2009). The situation is also complicated by the fact that serum Cr is insensitive to small changes in GFR (Slocum *et al.*, 2012). Hence, the precise time of insult may predate GFR decline and subsequent blood Cr elevations by several hours or longer (Portilla *et al.*, 2008). In the clinical setting, this implies that blood Cr concentrations could be "normal" in the face of AKI and reduced GFR, leading to misdiagnosis, delayed treatments and the added risk of progression to acute tubular necrosis with its associated complications.

1.3.3. *Novel biomarkers of AKI.* From the above discussion, it is clear that the current AKIN criteria utilises changes in Cr and urine output as surrogates for an AKI despite these being insensitive, non-specific and late markers of disease (Slocum *et al.*, 2012). Consequently, there has been intense research over the past decade to discover biomarkers reflective of kidney cell injury that could aid early diagnosis and treatment of AKI. This process of identification is costly and has recently involved use of spectrometry-based proteomic and metabolomic analyses as they have the advantage of utilizing blood and urine (Wang *et al.*, 2010). So far, this has yielded a number of potential biomarkers such as interleukin-18, kidney injury molecule-1, liver fatty acid binding protein-1 and cystatin-C - the latter of which is not a kidney injury biomarker *per se,* but as a filtration biomarker demonstrates an earlier rise compared to Cr when detecting falls in GFR (Han *et al.*, 2002; Herget-Rosenthal *et al.*, 2004; Ichimura *et al.*, 2004; Parikh *et al.*, 2004; Yamamoto *et al.*, 2007; Wu *et al.*, 2008).

At the time of commencing this doctorate, of the numerous potential AKI biomarker candidates, neutrophil gelatinase-associated lipocalin (NGAL) has shown the most promise (Carrillo-Esper *et al.*, 2011). NGAL is a 25 kDa protein encoded by the *LCN2* gene. Originally isolated from neutrophil granules it is secreted by the epithelia of numerous organs where its functions include limiting bacterial growth by sequestering iron-containing siderophores (Flo *et al.*, 2004; Mishra *et al.*, 2005; Wang *et al.*, 2010). It also participates in the motility of breast carcinoma cells and has been classed as a cytoprotective factor in conditions of oxidative stress (Roudkenar *et al.*, 2008; Fougère *et al.*, 2010). In the setting of AKI however, NGAL is up-regulated in high concentrations in the blood and urine. Rodent models of ischaemic AKI suggest the source of release to include the proximal and distal renal tubules (Mishra *et al.*, 2003; Paragas *et al.*, 2011). In humans, NGAL is elevated within two hours of renal insult and numerous studies have demonstrated superior sensitivity and specificity for AKI compared to blood creatinine in a variety of clinical settings e.g. intensive

care, emergency room, post-cardiac surgery and post-kidney transplantation (figure 1.12.; Mishra *et al.*, 2003; Mishra *et al.*, 2006; Nickolas *et al.*, 2008; de Geus *et al.*, 2011; Parikh *et al.*, 2011).

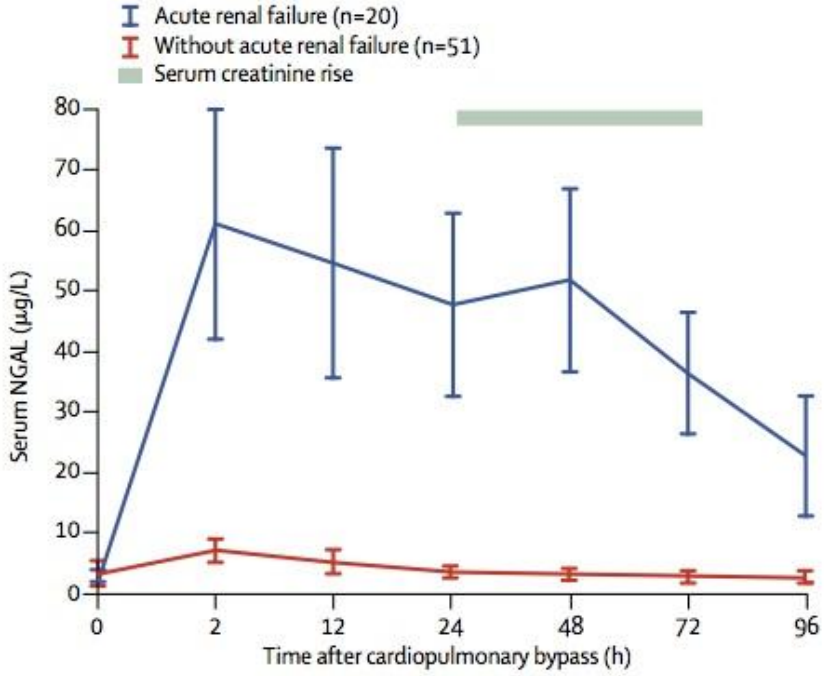

Figure 1.12. One of the seminal studies demonstrating the clinical utility of NGAL as an AKI biomarker in children who underwent open-heart surgery with cardiopulmonary bypass. In the 20 patients who sustained AKI (N = 20), serum NGAL concentrations were elevated at two hours (P = 0.001) whereas those without AKI (N = 51) did not demonstrate any discernable rise (P > 0.05). Those with AKI had serum creatinine elevations (> 50% from baseline) 22 hours after NGAL. Adopted from Mishra *et al.*, 2005.

Furthermore, NGAL elevations are proportional to the severity of the AKI. This has prognostic value with high concentrations associated with a greater incidence of renal replacement therapy and in-hospital mortality, both in the presence and the absence of a blood Cr rise (Hasse *et al.*, 2011). Such favorable characteristics have led to the development and availability of turbimetric and rapid point-of-care NGAL immunoassay kits for clinical use, but at present the cost-effectiveness of this approach remains debatable (Han *et al.*, 2009; Koch *et al.*, 2011).

1.4. *DOES AKI OCCUR DURING STRENUOUS PHYSICAL ACTIVITY?*

At the very least, the numerous changes to kidney physiology during heavy exercise calls into question whether a kidney injury (albeit temporary) is occurring. The bulk of laboratory-based and observational work examining kidney function during exercise predates current AKIN criteria. However, if AKIN criteria were applied, they would satisfy stage one serum creatinine elevations in many instances, especially during endurance-based activity (Neumayr *et al.*, 2003; Mingels *et al.*, 2009). An AKI in this setting seems plausible as the aetiology of the majority (> 70%) of AKI cases encountered in the clinical setting are pre-renal i.e. involving a sudden reduction in RBF (Hou *et al.*, 1983). This and falls in GFR are phenomena clearly shared with strenuous exercise.

However, there are some caveats. Firstly, in the majority of cases, urine flow rates does not usually remain depressed for more than six hours post-exercise (the time period of oliguria as stated in AKIN criteria). Secondly, increases in blood Cr may be confounded by increased muscle damage sustained through exercise, combined with the effect of a decrease in GFR. Thirdly, as mentioned earlier, the use of estimated GFR to document acute variation in renal function during exercise could be flawed, as such equations are derived from populations with CKD and are not valid in acute settings such as AKI and other experimental circumstances where there is brief manipulation of kidney function. Indeed, there is suggestion that GFR criteria should be developed for athletes as these may significantly differ between different disciplines depending on the phenotype required for the sport e.g. rowers versus rugby players (Lippi *et al.*, 2011). Finally, although GFR falls during intensive exercise, this is maintained with no further decrements if exercise is performed over a longer period at a similar intensity (Poortmans *et al*., 1996). This is not the case in instances of AKI requiring hospital admission, where GFR may continue to decrease, especially if the stressor is prolonged or maintained e.g. by infection and / or nephrotoxic medication use (Nash *et al.*, 2002).

Few studies have investigated the application of molecular AKI biomarkers, such as NGAL, following exercise. These have been observational and focussing only on endurance activity and not the high-intensity exercise which elicits more profound changes in kidney physiology (McCullough *et al.*, 2011). Additionally, since endurance events can last several hours, use of these makes it difficult to determine how early NGAL is up-regulated by exercise. Additionally, urinary concentrating effects have not been taken into account and little comparisons has been made to other traditional biomarkers of kidney injury. NGAL

concentrations in both blood and urine are elevated post-event but are short-lived and so far do not approach ranges associated with AKI as seen in clinical settings (McCullough *et al.,* 2011). Thus, there is considerable scope here for further research.

Regardless, in the real-world setting, most healthy people who regularly perform strenuous exercise will not be labelled with a diagnosis of AKI, because: 1) physiological changes to kidney function during exercise are considered by the medical community to be "normal" and temporary; and 2) most individuals do not come to the attention of a clinician for further investigation following heavy exercise unless it is incidental or a harmful event occurs e.g. collapse post-marathon. There are nonetheless specific instances following heavy physical exertion where a prolonged derangement to kidney function arises that becomes directly responsible for hospitalisation, intensive monitoring and specific therapies that may extend towards a severity requiring dialysis and fulfilling stage three AKIN criteria (Mehta *et al.*, 2007). The outcomes here are not always favourable and death can result, particularly when multi-organ failure ensues. These will now be discussed.

1.4.1. *Exertional rhabdomyolysis.*

1.4.1.1. *Definition.* Exertional rhabdomyolysis represents the rapid breakdown of striated muscle through strenuous physical activity (Cervellin *et al.*, 2010). Following muscle fibre necrosis, leakage of intracellular calcium activates cellular proteases with the release of numerous intracellular muscle proteins such as myoglobin, creatine kinase and lactate dehydrogenase (Warren *et al.*, 2002). Apart from well-recognised symptoms such as delayed-onset muscle pain, soreness and weakness, AKI associated with rhabdomyolysis is a life-threatening complication (Bywaters and Beall, 1941).

1.4.1.2. *Incidence.* There is a variable incidence of AKI associated with rhabomyolysis due to the different settings which this is experienced, but estimates range from 13 up to 50% (Bosch *et al.,* 2009). In a law enforcement training class where 50 participants exhibited laboratory evidence of rhabdomyolysis, 13 (26%) were admitted to hospital with evidence of "renal failure" (serum Cr > 176.8 mmol/l): of which, six received dialysis and one died of kidney and liver failure from combined rhabdomyolysis and heat stroke (MMWR, 1990). In contrast, cases appear to be infrequent in marathon runners. Between 1969 and 1986, Seedat *et al.* (1990) reported only 19 cases of acute renal failure reported in the South African Comrades marathon; even when participants were averaging 2,000 to 10,000 per year over this time

period. There are now probably fewer reports in the literature of this occurrence as it is no longer considered novel and there is greater awareness of precautionary measures (Clarkson 2007).

1.4.1.3. *Pathophysiology.* Overwhelming myoglobin precipitation within renal tubules is the principle cause of AKI following exertional rhabdomyolysis. Myoglobin is a single peptide protein of 153 amino acids with a molecular weight of 17.8 kDa (Kagen 1979). A small (0.01-5%) but constant proportion of filtered myoglobin is excreted into the urine under resting conditions and most is reabsorbed by the proximal tubules where it is catabolised though endocytosis and proteolysis (Beetham 2000). However, in exertional rhabdomyolysis the filtered myoglobin load can exceed the reabsorptive capacity of the tubule, and myoglobinuria (cola-coloured urine) can result once its concentration surpasses 100 mg/dl (or plasma concentration > 1.5 mg/dl) (Knochel 1982).

There are two key factors that promote myoglobin precipitation. Firstly, a low urinary pH (< 5.6) is likely to cause myoglobin to dissociate to globulin and toxic haematin (Ellenhorn, 1997). In murine models, acidic urine is required to promote renal impairment when myoglobin concentrate is intravenously infused (Zager 1989). This leads to several potential mechanisms of injury, including renal vasoconstriction by activation of the cytokine cascade, intraluminal cast formation and retention with Tamm-Horsfall protein which physically blocks tubules (Zager 1996). There is also evidence for direct haem-protein induced cytotoxicity through free-radical formation (Salahudeen *et al.*, 1996). The second factor is hypovolaemia which impacts upon RBF and promotes reduced urinary flow rates, enabling myoglobin to remain within tubules and exert toxicity (Beetham 2000).

1.4.1.4. *Diagnosis.* To determine the extent of rhabdomyolysis, usually blood creatine kinase (skeletal muscle isoform) is measured since it is regarded as the most sensitive measure of myocyte injury (Moghtader *et al.*, 1997). Blood creatine kinase concentrations in humans are typically 45-260 U/l and elevations due to rhabdomyolysis typically occur within 12 hours from the onset of muscle injury, peaking at one to three days post, and declining after three to five days (Huerta-Alardin *et al.*, 2005). In exercise involving a significant eccentric component, substantial rises in creatine kinase are expected (Clarkson and Hubal, 2002). Additionally, exercise intensity rather than duration is the most important determining factor for muscle enzyme release as endurance-type exercise generates creatine kinase concentrations < 5000 U/l and peak approximately 24 hours post-exercise (Clarkson 2007).

While myoglobin is a direct marker of muscle damage, it is rarely used in the diagnosis of rhabdomyolysis. Firstly, myoglobin concentrations may not fully reflect progressive muscle damage given its shorter half-like in plasma compared to creatine kinase (two to three hours vs. 1.5 days, respectively) (Poels and Gabreels, 1993). Also, myoglobin is rapidly metabolised to bilirubin and excreted in the urine with concentrations returning to normal by 24 hours, thus making it difficult to monitor the evolution of muscle damage (figure 1.13, Huerta-Alardin *et al.*, 2005).

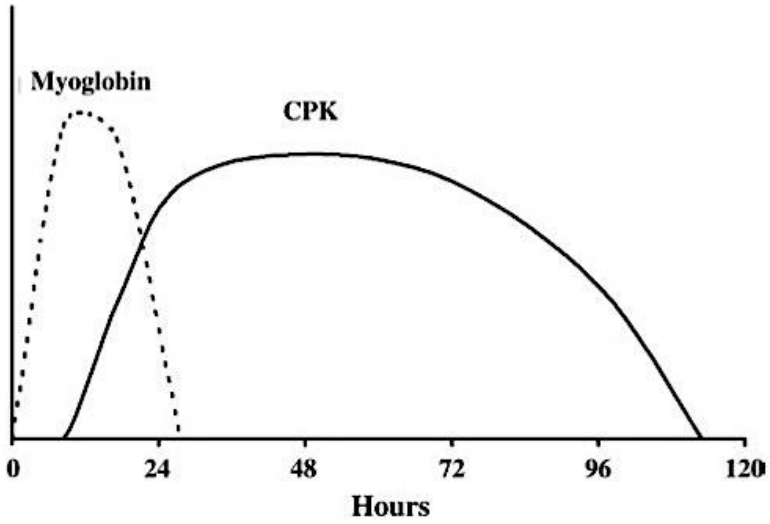

Figure 1.13. Kinetics of plasma myoglobin and creatine kinase (CPK) elevations following rhabdomyolysis. Myoglobin is the first protein to increase, but owing to its rapid clearance from plasma concentrations, rapidly return to normal within 24 hours. Conversely, CPK is elevated a few hours later than myoglobin but reaches its peak value within the first 24 hours and can remain as such for up to three days. This characteristic enables CPK to be a more practical marker for the severity and evolution of muscle injury. Adopted from Giannoglou *et al.*, 2007.

The creatine kinase concentration at which AKI is seen with exertional rhabdomyolysis is not well defined. Overall, there appears to be a poor correlation between creatine kinase and plasma Cr concentrations (Clarkson and Eichner, 2006). Very high creatine kinase concentrations may be encountered without any significant rise in plasma Cr or fall in urine output (Clarkson et al., 2006). Sinert *et al.* (1994) reported on 35 cases of exertional rhabdomyolysis with mean admission creatine kinase of 40,471 U/l though a retrospective chart analysis over a five-year period. None of their cohort developed renal impairment (as defined by plasma Cr > 2 mg/dl or urea > 25 mg/dl) or other related metabolic consequences such as hyperkalaemia, hyperphosphataemia, hypocalcaemia, or acidosis. However, if the AKIN criteria are applied then six out of their 35 patient cohort would have satisfied stage

one AKI (Mehta *et al.*, 2007). Another study of 477 rhabdomyolysis cases found a highly significant association between serum Cr and creatine kinase concentrations ($R^2 = 0.12$, $P < 0.0001$), but the predictive value of creatine kinase determining the variance of serum Cr in this model was low and there were only three exertional rhabdomyolysis cases noted over an eight year period (Melli *et al.*, 2005). A previous study has also confirmed this lack of predictive correlation (Gabow *et al.*, 1982).

Such findings have contributed to the lack of clear guidance to when individuals, based on creatine kinase concentrations, should be monitored and hospitalised for the potential development of AKI (Clarkson and Eichner, 2006). Nonetheless, most authorities advocate that creatine kinase concentrations of greater than five to ten-times the upper limit of normal or between 5,000 to 10,000 U/l should at least warrant observation of urine output, myoglobinuria and daily measurement of plasma Cr, whereas treatment should be instigated prophylactically at 20,000 U/l (Huerta-Alardin *et al.*, 2005; Terpilowski and Cridddle, 2004; Clarkson and Eichner, 2006a, Cervellin *et al.*, 2010).

Urinary concentrations of myoglobin have been investigated as alternative predictors of AKI onset (Rodriguez-Capote *et al.*, 2009). However, this has been hampered by the reasons discussed above regarding myoglobin analysis and the paucity of well-designed trials. A recent systematic review concluded that the sensitivity of urine myoglobin in patients with AKI due to rhabdomyolysis was 100% from eight studies, but specificity was much lower and varied between 15-91%. This was partly due to marked differences in study design, disease spectrum, reference standard and test methodology (Rodriguez-Capote *et al.*, 2009). There is a suggestion that serum myoglobin together with a low urine myoglobin (indicating low clearance) may indicate a high risk for developing AKI in the clinical setting, but the cost-effectiveness of this approach is doubtful (Wu *et al.*, 1994a; Rodriguez-Capote *et al.*, 2009). Moreover, in the context of physical activity, serum myoglobin concentrations do not necessarily correlate with the occurrence of kidney injury: after a 99 km ultramarathon 25 out of 44 runners had elevated serum myoglobin but none developed kidney injury (Schiff *et al.*, 1978). The usefulness of other muscle damage markers and their relationship with AKI has not been established to justify their routine clinical use (Cervellin *et al.*, 2010).

Given the variability in the relationship between biomarkers of muscle damage to predict AKI, it is clear that other factors must be involved. These include: a lack of preconditioning to strenuous exertion, heat stress, preceding viral infection, use of non-steroidal anti-inflammatory drugs, alcohol, cocaine, heroin, hyperuricaemia, diuretics, latent myopathy (e.g. McArdle's syndrome), urate metabolism, sickle cell trait and possibly a genetic predisposition

involving polymorphisms of the myosin light chain kinase gene (Huerta-Alardin *et al.*, 2005; Clarkson *et al.*, 2006b). The latter may be particularly significant as there is wide inter-individual variability in the magnitude and time course of plasma creatine kinase response related to exercise-induced muscle damage, and this in turn leads to a classification of low, medium and high creatine kinase responders (Chen 2006). Thus, it is likely that a "perfect storm" of factors may be required for an AKI to develop (Clarkson 2007).

1.4.1.5. *Treatment and prognosis of AKI in exertional rhabomyolysis.* Treatment of AKI from exertional rhabomyolysis primarily involves aggressive intravenous overhydration (Huerta-Alardin *et al.*, 2005). A forced diuresis when started within six hours of diagnosis has been reported to minimize the risk of AKI developing (Zager 1996). The choice of intravenous fluid is currently debatable. Given the evidence of myoglobin precipitation in renal tubules at a low urinary pH, dilute sodium bicarbonate (e.g. 1.26%) solutions are advocated to achieve alkaline urine (pH above 7.5) and a solute diuresis (Eneas *et al.*, 1979; Ozgüç *et al.*, 2005). But despite its widespread use, superiority to 0.9% saline solutions is so far lacking (Homsi *et al.*, 1997; Brown *et al.*, 2004; Huerta-Alardin *et al.*, 2005; Scharman and Troutman, 2013). There are theoretical benefits of diuretics such as mannitol and furosemide that would maintain urine flow rates and reduce the likelihood of tubular myoglobin deposition, but good randomised controlled data is lacking (Karajala *et al.*, 2009).

In the majority of cases, the above treatments result in resolution of AKI within days (Huerta-Alardin *et al.*, 2005; Cervellin *et al.*, 2010). Occasionally, damage to tubules may be more extensive and result in an acute tubular necrosis characterised by increasing blood creatinine and urea, variable urine output, progressive metabolic acidosis and severe hyperkalaemia despite intravenous fluids. In such instances, prolonged supportive treatment dialysis may be required for weeks to months, but despite this the outcome for complete recovery in renal function remains good (Uberoi *et al.*, 1991). With respect to preventative strategies, modifiable risk factors should be addressed e.g. good hydration prior to exercise, limited use of non-steroidal anti-inflammatory drugs, and pre-conditioning, which is known to reduce myoglobin release from myocytes after exercise (Byrnes *et al.*, 1985).

1.4.2. *Exertional Heat Stroke*

1.4.2.1. *Definition.* Exertional heat stroke is a life-threatening condition where central nervous system abnormalities (e.g. confusion, seizures, coma) occur following physical activity in hot

environments where excessive heat generated from cannot be dissipated effectively (Bouchama and Knochel, 2002). A core body temperature of typically > 40^{o}C has often been stated as being part of the definition, but lower values satisfying hyperthermia (i.e. > 37.5^{o}C) may also be associated with heat stroke. Other organ dysfunction may also develop and this includes AKI, which may or may not be associated with exertional rhabdomyolysis (Schrier *et al.*, 1967; Schrier *et al.* 1970; Raju *et al.* 1973).

1.4.2.2. *Incidence.* The incidence of AKI in exertional heat stroke appears to be variable and depends upon the population studied and the presence of risk factors such as sex (women > men), geographical region of origin (hotter > cooler climates) and ethnicity (Caucasians > Afro-Carribean) (Carter *et al.*, 2005). Incidence rates are better described in the military literature and have varied between 10-25%. This is partly reflective of the various definitions of heat stroke employed (Schrier *et al.*, 1967; Clowes and O'Donnell, 1974; Chapman 1995). In one of the largest epidemiological studies of heat illness in US military personnel involving 5246 soldiers, Carter and colleagues placed the incidence of AKI in heat stroke at 16% (Carter *et al.*, 2005). However, once heat stroke victims are admitted into the intensive care unit, AKI almost invariably seen within 24 hours of presentation (Pease *et al.*, 2009).

1.4.2.3. *Pathophysiology.* The kidneys act as both victim and villain to the multi-organ dysfunction that typifies exertional heat stroke. The pathophysiology is complex and is believed to involve interplay between heat cytotoxicity, disseminated intravascular coagulation, and a systemic inflammatory response syndrome (Perchick *et al.*, 1975; Shieh *et al.*, 1995; Willatts *et al.*, 1995; Heled *et al.*, 2013).

Hyperthermia may lessen cell survival through a direct effect on membrane integrity, increased enzymatic reactions rates, and generation of harmful reactive oxygen species (Leon and Helwig, 2010). However, this may vary amongst individuals as conditioned athletes can tolerate hyperthermia without side-effects due to the training-induced heat acclimatization effects on cellular protective mechanisms (McClung *et al.*, 2008). Furthermore, the increase in skin blood flow in hyperthermic states is accompanied by a compensatory fall in splanchnic blood flow to maintain blood pressure (Nybo, 2007). Such haemodynamic alterations could compromise the kidneys further when already up to 50% of RBF is redirected during intense exercise (Walker *et al.*, 1994).

Thermal injury to the vascular endothelium is regarded as the primary initiating event that leads to disseminated intravascular coagulation (Mustafa *et al.*, 1985). *In vitro* studies

have shown that exposure to temperatures of 43-44^{o}C causes platelet activation and aggregation (Gader *et al.*, 1990). Together with the excess deposition of fibrin, this leads to microvascular thromobosis. Organ dysfunction ultimately results and hypoperfusion is further exacerbated by blood loss due to excessive intravascular consumption of coagulation products. Compounding this, endotoxin can activate cytokines and endothelial cells to amplify the coagulation cascade (Huisse *et al.*, 2008).

The contribution from systemic inflammatory response syndrome in heat stroke is thought to result from the translocation of gastrointestinal bacteria into the systemic circulation when splanchnic blood flow to the gut is compromised (Leon and Helwig, 2010). This endotoxaemia in turn activates numerous pro- and anti-inflammatory intracellular peptides including cytokines interleukin (IL)-1α, IL-1β, IL-1 receptor antagonist, IL-6, soluble IL-6 receptor, IL-8, IL-10, IL-12, interferon-γ, tumor necrosis factor-α; all of which are commonly observed following collapse from heat stroke (Leon 2007). The function of these molecules is very complex and different interactions are observed depending upon the clinical or experimental model studied e.g. plasma IL-8 concentration is elevated in exertional heat stroke but unchanged following exercise in the heat (Heled *et al.*, 2013). Additionally, there may be different sources cytokine production. For example, IL-6 is secreted by active musculature but can also be derived from the renal tubules and epithelia in the setting of AKI with heat stroke (Lu *et al.*, 2004; Lee *et al.*, 2011; Pedersen 2013). Therefore, as a source of cytokine synthesis, the kidneys may serve to propagate the pathophysiological processes underlying heat stroke.

Environmental factors that contribute to exertional heat stroke evolution are many and include: a heat stressed environment, dehydration, obesity and over clothing (Adams *et al.*, 2012). A history of exertional heat illness, recent or current viral illness and fever have only been anecdotally associated. Genetic factors involving polymorphisms in genes that encode proteins associated with the acute phase response to heat could interact with environmental triggers leading to the development of organ dysfunction (Protasi *et al.*, 1995).

Aside from the cytokine milieu, circulating vasoconstrictive and vasodilatory hormones are present in exertional heat stroke. These are recognised to play a role in the pathogenesis of ischaemic and septic models of AKI in both humans and animals (Cumming *et al.*, 1988). In brief, during the acute phase of exertional heat stroke, marked elevations of renin, aldosterone, plasma catecholamines and endothelin-1 result in a predominantly vasoconstrictive environment in the kidney (Lin *et al.*, 2003). However, the concurrent reductions in prostaglandin E_2 with rise in nitric oxide concentrations fail to counterbalance

this. The resultant renal haemodynamic status is likely to lead to extensive endothelial injury and tubulointerstitial damage to the kidneys. Co-existent rhabdomyolysis can also exacerbate the clinical picture (Shieh *et al.*, 1992). Lin *et al.* (2003) attempted to summarise the factors and complex interactions that lead to AKI during exertional heat stroke (figure 1.14).

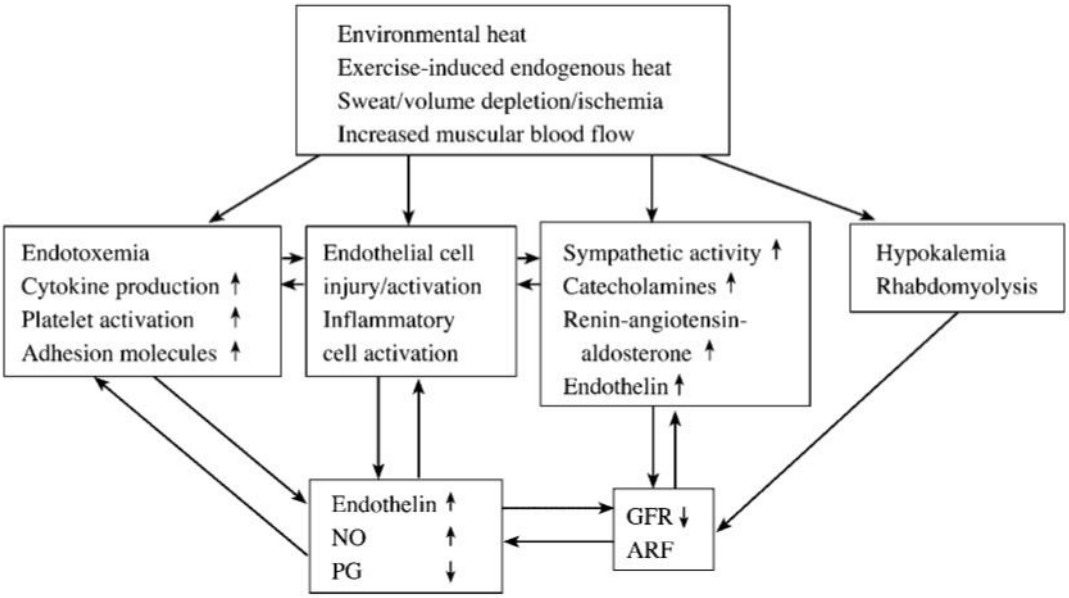

Figure 1.14. A proposed mechanism elucidating the complex relationship between vasoactive mediators, inflammatory responses and renal haemodynamics in exertional heat stroke. NO: nitric oxide; PG, prostaglandin; GFR, glomerular filtration rate; ARF, acute renal failure i.e. AKI. Adopted from Lin *et al.*, 2003.

1.4.2.4. *Diagnosis*. Hyperthermia can be reliably assessed by using an oesophageal or rectal probe that determines core temperature, and central nervous system abnormalities can be defined by the Glasgow Coma Scale or characterised by seizure activity (Adams *et al.*, 2012). Following this, heat stroke victims need to be observed assiduously for development of organ dysfunction. This can be monitored through regular assessment of blood biomarkers concentrations. However, these may not be sensitive or specific enough to detect damage as they can also be released from other organs and tissues altered by heat and exhaustive exercise e.g. up-regulation of creatine kinase may suggest rhabdomyolysis or myocardial infarction (Goddard and Warnes, 1992; Wallimann and Hemmer, 1994). In addition, there is suggestion that over-reliance of these traditional biomarkers may result in misdiagnosis of heat stroke and its recovery: rat models of heat stroke show dissociation between temperature profiles, urea, aspartate transaminase and alanine transaminase, with histological damage to kidney and liver at 10 days post-heat stroke recovery (Leon and Helwig, 2010). This implies that traditional biomarkers expected to be reflective of organ damage in heat stroke are not

specific enough and strengethens calls for investigation of novel biomarkers in heat-stroke mediated organ injury, such as NGAL.

1.4.2.5. *Treatment and prognosis.* Apart from immediate resuscitative measures, rapid whole-body ice-cold water immersion is one of the recognised evidence-based treatments for lowering core temperature and preventing the downstream effects on thermal injury upon organ function (Smith 2005; Casa *et al.*, 2012). Others include evaporative cooling, ice cold packs in axillae and more invasive procedures such as gastric and peritoneal ice lavage. There is no role for dantrolene and anti-pyretic agents such as paracetamol, and no randomised-controlled trials have assessed the effectiveness of non-steroidal anti-inflammatory drugs (Smith 2005; Leon and Helwig, 2010). If immersion is delayed, the risk of permanent organ damage is increased (Bouchama and Knochel, 2002). Once multi-organ failure ensues, supportive management in the intensive care setting e.g. invasive ventilation techniques and vasopressors, correction of coagulation derangements and renal replacement therapy. Compared to standard haemofiltration, trials utilising high cut-off haemofiltration techniques or plasma exchange may be beneficial for the removal of cytokines involved in heat stroke (Raj *et al.*, 2013; Atan *et al.*, 2013).

Potential therapies for severe sepsis have also been trialled in heat stroke. These include anti-cytotoxin, anti-endotoxin and anti-coagulation therapies, but results are not promising and multicentre trials involving over 6000 severe sepsis patients have shown no benefit on all-cause mortality (Redl *et al.*, 1998). There are several reasons for this including limited understanding of the precise roles of cytokines in heat stroke, failure to neutralise target proteins, compensatory increase of other mediators with similar activities, and inappropriate timing or duration of therapy (Remick, 2003). The latter may be relevant in terms of the lack of ability to precisely define the start of organ injury, and again injury specific biomarkers such as NGAL could assist in this regard and lead to re-evaluation of promising cytokine-targeted treatment.

Multi-organ failure in heat stroke carries a mortality of greater than 20% (Casa *et al.*, 2012). Additionally, 30% of survivors experience permanent reductions in organ or tissue function that in turn increase the risk of mortality during the subsequent recovery period (Argaud *et al.*, 2007). Military heat stroke patients have also shown a two-fold increased mortality from cardiovascular risk, kidney, and liver failure within 30 years of hospitalisation for a non-heat related illness (Wallace *et al.*, 2007). Heat stroke is currently more preventable than treatable. Strategies include acclimiatization to heat, reduction in duration and intensity

of physical activity, rescheduling of activities to cooler times of the day, adequate hydration, appropriate clothing, and removing vulnerable populations from the heat stressed environment, e.g. those with an infection (Leon and Helwig, 2010; Adams *et al.*, 2012).

1.4.3. Acute Renal Failure with Severe Loin Pain and Patchy Renal Ischaemia after Anaerobic Exercise.

1.4.3.1. *Definition.* This is a less well-recognised form of AKI that occurs following anaerobic exercise e.g. short track sprints. Symptoms are characterised by a prodrome of nausea and vomiting followed by loin or abdominal pain that is typically sharp and lancinating in nature (Ishikawa *et al.*, 1982). The pain is thought to be due to vasospasm (renal angina), but given the condition's association with use of analgesics, flank pain due to concurrent use of non-steroidal anti-inflammatory drugs and papillary necrosis may be difficult to distinguish (Johnson and Wen, 1995). The patchy renal ischaemia refers to the typical appearance of contrast enhancement following computer-aided tomography imaging (Ishikawa *et al.*, 1981).

1.4.3.2. *Incidence.* Less than 200 cases have been reported in the literature so far, with over 90% occurring in young males (Ishikawa 2002). There also appears to be a preponderance of this condition in Japan, possibly a reflection of increased awareness and recognition, and hence, diagnosis, of the syndrome in Japan.

1.4.3.3. *Pathophysiology*. It is believed that during anaerobic exercise, the rapid re-diversion of blood flow to musculature is responsible for significant kidney ischaemia and acute loin pain (Ishikawa 2002). However, as discussed above, this is a physiological adaptation that would not normally cause clinically significant ischaemia. Therefore, an additional insult must be necessary. Given that approximately half of affected individuals have a low serum uric acid concentration together with a high fractional excretion of urinary uric acid, renal hypouricaemia has a likely pathological role (Ishikawa 2002). The lack of uric acid through its increased excretion prevents the neutralisation of reactive oxygen species that are formed during anaerobic activity (Murakami 1995). Such free radicals can inhibit the action of cyclooxygenase, which catalyses a critical step in the production of Prostaglandin E_2, and as mentioned is an important mediator of renal vasodilation during times of reduced RBF (section 1.1.1.). In recent years, this purported mechanism has gained support from genetic

studies of affected individuals that revealed mutations in the urate anion exchange gene 1 that is responsible for increased urate excretion (Tanaka *et al.*, 2003). A previous theory of a uric acid crystallopathy leading to tubular obstruction is not supported by renal biopsies that instead consistently demonstrate an acute tubular necrosis (Ishikawa 2002).

1.4.3.4. *Diagnosis*. Apart from biochemical evidence of kidney injury, a key diagnostic feature is patchy wedge-type enhancement of the kidneys following contrast-aided computer tomography (figure 1.15). This reveals itself in a delayed fashion over hours to a few days and the contrast enhancement appears to be more diffuse when the AKI is more severe (Ishikawa 2002).

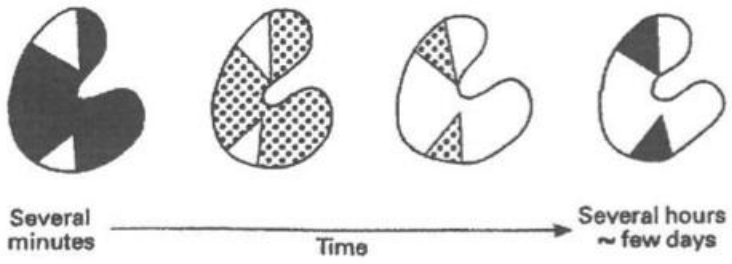

Figure 1.15. A schematic illustration of wedge-shaped contrast enhancement following acute renal failure with severe loin pain and patchy renal ischaemia after anaerobic exercise. Initially, contrast media is not taken up by the wedge-shaped diseased area but appears later on. The size of the wedge-shape appears to be proportional to the degree of kidney injury. Adopted from Ishikawa, 2002.

Furthermore, creatine kinase and myoglobin only demonstrate mild elevations in serum (upper normal of creatine kinase and myoglobin less than seven and nine-times upper limits, respectively) (Ishikawa 2002). These lower elevations are thought to be in part due to the predominantly type II muscle fibre damage that ensues following performing anaerobic exercise bouts (repeated short sprints); type II fibre having lower amounts of myoglobin than type I fibres (Berchtold *et al.*, 2000).

1.4.3.5. *Treatment and prognosis.* AKI in this condition is typically mild and treated with conservative measures (e.g. intravenous fluids), although cases requiring dialysis have been reported (Ishikawa 2002). Given the non-oliguric nature of this AKI, prognosis appears to be good in most cases despite dialysis, but in a significant proportion (around 20%), this condition recurs on performing exercise (Ishikawa 2002).

1.5. *AIMS OF PHD STUDY*

In the preceding sections, the key alterations to kidney physiology that occur through heavy physical exercise has been described in detail and their stressful nature appears to resemble pathological states. The discussion then progressed to defining AKI and attempting to apply this to the marked perturbations that the kidneys experience during exercise. Finally, taking a step further into the clinical setting, three specific instances where severe AKI is encountered in exercise were described in detail. The clinical significance of these settings highlights the need for further research to dissect the interactions between exertional activity and AKI. Understanding why apparently healthy individuals can rapidly develop life-threatening pathology may have far-reaching public health implications and permit prediction of individuals who are at risk. To this end, the subsequent four chapters represent studies exploring specific areas of this relationship.

Chapter 2 aims to determine if the presence of post-exercise proteinuria indicates a mild transient exercise-induced kidney injury. This is conceivable as there is physiological stress during exercise and proteinuria *per se* is known to be damaging to the kidney (section 1.1.4.). Through a short duration but high-intensity bout of proteinuria-inducing exercise, a biomarker of kidney injury (NGAL) is measured post-exercise to determine whether kidney injury or stress occurs. Such efforts would allow further insights into site-specific renal stresses during exercise and may provide a novel and more sensitive method of assessing the acute effects of exercise on the kidney than blood Cr. Additionally, any changes in NGAL concentrations post-exercise may have implications in biomarker interpretation within the clinical environment (cf. troponin, section 1.2.1.).

Further investigation of post-exercise proteinuria is pursued in **chapter 3.** Using a systematic review of available literature, the acute phenomena of post-exercise proteinuria is explored as a risk factor for the onset or progression of CKD. Persistent proteinuria is a biomarker of CKD, and is pathogenic if left untreated i.e. it can accelerate the progression of CKD. However, there is currently no consensus on whether exercise-induced proteinuria represents a potentially useful test to predict the onset or progression of CKD. This is an important question as such a diagnostic tool could potentially provide a non-invasive method of determining the onset of nephropathies at various stages, or act as an adjunctive or triage assessment before more risky invasive procedures such as renal biopsy. Additionally, it could allow for early proactive measures to prevent disease progression. Previous work in this area is scarce and has generally focused on diabetics with or without nephropathy.

By using an exercise-induced muscle damage model followed by heat stress, **chapter 4** probes the inflammatory component of AKI. Exercise-induced muscle damage, heat stress and AKI are each typified by a pro-inflammatory state. Moreover, the cytokine IL-6 is released by exercising muscle and also plays a crucial role in the initiation and development of inflammation in AKI. By exposing healthy individuals to a prior bout of muscle-damaging exercise followed with endurance exercise in the heat, novel and traditional AKI biomarkers are measured to determine whether increased kidney stress occurs. Such findings would have implications for at-risk populations including amateur and professional athletes, military personnel and firefighters, as well as offering insights into the pathophysiology of heat illness and AKI.

Chapter 5 attempts to further isolate the relationship between plasma IL-6 and biomarkers of kidney injury and function. Despite its perceived importance as a mediator of AKI, the experimental manipulation of IL-6 in animal models yields variable findings depending on type of AKI studied. Such disparities could be due to variable degrees of insult, experimental time course, definitions of AKI used, or be reflective of pleiotropism exhibited by IL-6. The impact of IL-6, in humans upon biomarkers of AKI is currently unknown. Hence, recombinant IL-6 is infused into healthy humans to simulate concentrations experienced in an ischaemic model of AKI, and assessed the behaviour of biomarkers of kidney injury and function.

Finally**, chapter 6** concludes the thesis with a summary of the key findings arising from the four studies. Links are made between the studies and current literature. Aside from discussing the limitations of the methodologies used in the studies, clinical applications and future directions of research are also discussed.

CHAPTER 2

DOES PROTEINURIA-INDUCING PHYSICAL ACTIVITY INCREASE BIOMARKERS OF ACUTE KIDNEY INJURY?

2.1. *ABSTRACT*

It was determined if an acute kidney injury biomarker, neutrophil gelatinase-associated lipocalin (NGAL), would be up-regulated by high intensity proteinuria-inducing exercise. Through a prospective cohort design, 90 healthy, active adults (mean age 24 ± 4 (SD) years) were screened for post-exercise proteinuria (PeP); 10 PeP positive and 10 PeP negative participants then completed a high-intensity exercise protocol involving an 800 metre sprint. Plasma and urinary NGAL, urinary creatinine, urinary albumin and urine volume were obtained at the following time points: pre-run, immediately post-, 25 minutes, one hour and two hours post-run. Following maximal exercise, 64% of participants had urinary NGAL concentrations above the normal range, particularly at 25 minutes post (P = 0.002). However, there was no difference in NGAL response between PeP positive and negative groups and plasma NGAL was decreased, not elevated, following exercise (P = 0.002). In some individuals normalizing urinary NGAL for urinary creatinine attenuated elevations. Urinary NGAL was also negatively correlated with urine volume (r = -0.701, P = 0.005). In conclusion, proteinuria susceptibility did not influence an acute injury biomarker response to exercise. Nevertheless, urinary NGAL is likely to be elevated by exercise, possibly due to increased production by the proximal tubule, increased plasma clearance (given the decrease in plasma NGAL) and / or a concentrating effect of exercise-induced oliguria. Until correct normalisation of urinary biomarkers is determined, NGAL should be interpreted cautiously in exercise and acute kidney injury-induced oliguria. The inter-individual NGAL response to exercise also warrants further investigation.

2.2. *INTRODUCTION*

During exercise, the kidneys maintain homeostatic composition of extracellular fluid, thus enabling regulation of vascular volume and preservation of cardiac output. However, high-intensity physical activity is associated with marked perturbations in kidney function (Poortmans 1984). In fact, 30 - 40% decreases in kidney blood flow have been observed as blood is diverted to other metabolically demanding tissues such as the active musculature (Guyton and Hall, 2011). In addition, enhanced proteinuria and haematuria, reductions in glomerular filtration rate (GFR), impaired handling of electrolytes and amino acids are also apparent (Kadachorian and Johnson, 1970).

When chronic, such perturbations are usually associated with kidney pathology (Mookerje *et al.*, 2001). However, in exercise models these changes are interpreted as harmless as they typically resolve within 24 to 72 hours (Cianflocco *et al.*, 1992). Nevertheless, such derangements can indicate and progress to more serious acute kidney pathology. For example, in U.S. military members, acute kidney injury (AKI) / acute renal failure has been recognized as a significant threat during physical exertion, particularly under heat stress (Carter *et al.*, 2005). A delay in diagnosis of AKI is not uncommon and haemodialysis may be required.

One of the most commonly observed disturbances to kidney function with exercise is post-exercise proteinuria (PeP), but this shows large inter- and intra-individual variation (Poortmans *et al.*, 1982; Poortmans 1984, Poortmans and Vanderstraeten, 1994). Why such variability exists in renal responses to exercise, and why and some individuals progress to more serious kidney pathology remains unclear. Until recently, one obstacle has been a lack of sensitive biomarkers of kidney injury. Serum or plasma creatinine (Cr) is the most widely utilized measure of kidney function. At present, it forms the basis for a diagnosis of AKI and has also been repeatedly used to demonstrate the declines in renal function during exercise (Poortmans 1984; Ronco *et al.*, 2007). Despite its convenience, Cr is not a kidney injury biomarker *per se* and has its deficiencies such as a delayed response to an AKI (Waikar *et al.*, 2010). Thus, there has been avid interest in identifying molecular biomarkers which are capable of detecting kidney injury (and therefore impaired kidney function) much earlier than blood Cr. One promising biomarker is neutrophil gelatinase-associated lipocalin (NGAL; Mishra *et al.*, 2005). However, the behaviour of this and other novel biomarkers to exercise has only recently been examined (McCullough *et al.*, 2011). Responses to exercise protocols of varying intensity or duration remain unknown and whether individuals who are susceptible

to PeP also experience greater up-regulation in NGAL due to nephron-related stress has also not been determined.

Given the physiological aberrations to kidney function known to occur during exercise, their unexplained inter-individual response, and the recent development of more sensitive biomarkers of kidney injury, the aim of this study was to determine if NGAL is up-regulated following a short duration high-intensity bout of proteinuria-inducing exercise (800 metre sprint). In this model, it was hypothesized that: i) NGAL would show a significant but transient rise above normal ranges (but not approaching levels seen in AKI); and ii) the elevations would be greater in those with PeP compared to those without. If supported, this would reveal further insights into site-specific kidney stresses during exercise and provide a novel and more sensitive method of assessing the acute effects of exercise upon kidney function. Additionally, any elevations in NGAL post-exercise may have implications in biomarker interpretation within the clinical environment.

2.3. *MATERIALS AND METHODS*.

Study design and participants. An observational prospective cohort design was employed (figure 2.1.). In the first stage of the study, 90 potential participants (mean age ± SD, 24 ± 4 years; 38 female; 52 male) were approached via university and local sports clubs between September 2010 and July 2011 and were screened for presence of PeP. Subsequently, 10 PeP positive and 10 PeP negative participants, who were conveniently sampled and not purposefully matched, completed the second stage of the study. This involved collection of blood and urine samples following an experimental exercise protocol designed to induce proteinuria. All participants provided written informed consent and ethical approval was provided by the College of Health and Behavioural Sciences, Bangor University ethics committee.

Screening protocol for exercise proteinuria. All participants had no significant medical conditions nor took medications that could potentially influence kidney function (e.g. non-steroidal anti-inflammatory drugs). Screening for PeP was conducted through the following methods: rested individuals (i.e. who had not performed strenuous exercise in the last 72 hours) were asked to provide a urine sample prior to the start of their normal exercise routine. Approximately 30 minutes following this exercise a further urine sample was taken. Both samples were stored in ice boxes during transportation and auto-analysed for albumin:creatinine ratio (A:Cr) estimation within 24 hours of collection (both by Olympus AU2700 analyser; creatinine reagent OSR6178, Beckman Coulter, California, USA (kinetic Jaffe method); Microalbumin reagent MA2426, Randox Laboratories, Crumlin, UK). A baseline estimated GFR was also calculated by the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation (Levey *et al.*, 2009).

There are no official guidelines to the acceptable A:Cr limits for post-exercise proteinuria in healthy subjects, although literature exists regarding provocation of PeP in diabetics as part of a risk assessment of future diabetic nephropathy (Agarwal *et al.*, 1998). Therefore, to define whether an individual was PeP positive, the well-established criteria for significant microalbuminuria in diabetics was adopted and applied to the A:Cr difference between pre to post-exercise. Thus, participants were defined as PeP positive if their A:Cr rise was greater than 2.5 mg/mmol (male) or 3.5 mg/mmol (female; Jefferson *et al.*, 1985). Those not fulfilling such increases were deemed PeP negative.

Experimental protocol for proteinuria-inducing exercise. All PeP positive and randomly selected PeP negative participants were invited to complete the full experimental protocol.

Eventually twenty participants completed the protocol, performing an 800 metre sprint at the University athletic track between September 2010 and August 2011 (figure 2.1.). This form of high intensity, lactate-generating exercise is recognised as an optimal protocol for inducing PeP (Poortman *et al.*, 1996).

A day prior to any sampling of urine and blood, all participants were required to drink two litres of water (in addition to normal fluid intake) to ensure adequate hydration. Hydration status was assessed by using a portable meter (Pocket Pal-Osmocheck, Vitech Scientific, Partridge Green, UK) to assess urine osmolality immediately prior to completing the experimental protocol. If the value exceeded 700 mOsmols/KgH_20, individuals were asked to re-attend when adequately hydrated (Sawka *et al.*, 2007). Participants were also asked to refrain from heavy exercise for at least four days and not to eat during the two hours before testing.

Participants ran in groups of two or three to encourage competition and to elicit maximal effort. As a measure of exercise intensity (anaerobic energy production) during exercise, blood lactate was measured pre- and immediately post-run (Lactate Pro Lt-1710, Arkray, Kyoto, Japan; Jacobs *et al.*, 1986).

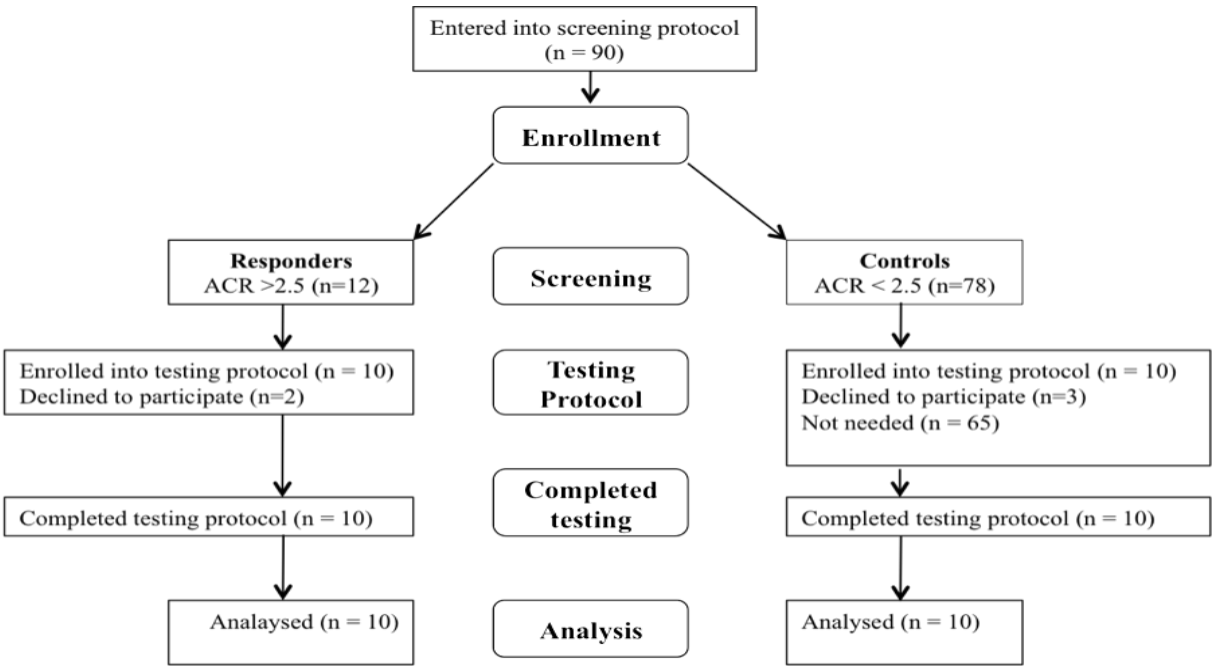

Figure 2.1. Study design flowchart. ACR, albumin:creatinine ratio.

Following the run, participants were rested. Venous blood and urine specimens were collected prior to, immediately post- (0 minutes), 25 minutes post-, one hour post- and two hour post-run. These time points were selected to maximise the chances of early detection of PeP and NGAL, as elevations in PeP have been shown to be maximal 20-30 minutes after exercise, while NGAL increases have been confirmed as early as two hours after the onset of AKI (Poortmans 1984; Mishra *et al.,* 2005). Urine volume was also recorded at each time point. Haemoglobin (β-Haemoglobin Hemocue AB photometer, Hemocue Ltd, Dronfield, UK) and haematocrit (Hawksley and sons Ltd, Sussex, UK) were also measured for subsequent plasma volume shift calculations (Dill and Costill, 1974). Following this, all blood specimens were centrifuged; plasma and urine aliquots were stored at -80ºC for subsequent biochemical analysis.

Ascertainment of albumin:creatinine and urine and plasma NGAL. A:Cr assessments were performed as described above. NGAL concentrations were determined using a commercially available enzyme-linked immunosorbent assay (NGAL Rapid ELISA, Bioporto, Gentofte, Denmark). Absorbances were read at a wavelength of 450 nm by a microplate reader (Opsys MR, Dynex Technologies, Worthing, UK). The normal ranges quoted by the kit manufacturer for urinary NGAL and plasma NGAL concentrations were 0.7-9.8 ng/ml and 37-106 ng/ml, respectively (NGAL, Bioporto Diagnostics). According to the kit insert, intra-assay variation was determined by measurement of NGAL in two urine and two plasma samples with eight replicates each. The coefficients of variation for both urine samples were 3.4% and 4.3%, and for plasma, 2.9% and 1.9%, respectively. Inter-assay variation was calculated through a similar process with two dilute urine and two dilute EDTA plasma samples in two replicates. The coefficients of variation for both urine samples were 4.7% and 22.7%, respectively, and for plasma, 11.4% and 12.4%.

As with A:Cr, urinary NGAL concentrations were corrected for urine Cr to account for changes in urinary concentration or dilution. Therefore, Cr corrected urinary NGAL (urinary NGAL:Cr) was expressed as ng NGAL / mmol urine Cr ($x10^{-3}$). Additionally, plasma NGAL concentrations were corrected for plasma volume change (Dill and Costill, 1974).

Statistical analysis. SPSS version 18.0 (IBM, Chicago, IL) was used for all statistical analysis. Statistical significance was accepted at $P \leq 0.05$. Data for all dependent variables were examined for normality through three methods: 1) Shapiro-Wilk tests; 2) skewness beyond the limits of +3 to -3; and 3) visual inspection of histogram plots. In the case of descriptive data and plasma NGAL, mean ± SD are stated. However urine volume, A:Cr, urinary NGAL and urinary NGAL:Cr were not normally distributed and remained markedly

skewed despite attempts at data transformation. Thus, these non-parametric data are presented as median {25th percentile, 75th percentile}. Nonetheless, in the interests of consistency, all results were graphically presented as boxplots.

Descriptive data were examined by Student's *t*-test. Correlations between variables were determined using Pearson's (r) or Spearman's rank (ρ) correlation coefficient, as appropriate. For plasma NGAL, to examine the interaction between proteinuria group (PeP positive or PeP negative) and time (pre, post 0 minutes, 25 minutes, 1 hour, and 2 hours-post) a parametric two-way repeated measures analysis of variance was used with *post hoc* Tukey's tests.

For urine volume, A:Cr, urinary NGAL and urinary NGAL:Cr a Friedman's test (a non-parametric alternative to repeated measures analysis of variance) was performed to examine the effect of time. The main effects of proteinuria group and the interaction between proteinuria group and time were examined using a two-way non-parametric analysis of covariance as outlined by Quade (Quade 1967). Significant differences were examined further using *post hoc* Wilcoxon Signed-Ranks with Bonferroni corrections for multiple comparisons.

For the primary outcome measure of urinary NGAL, a power calculation revealed that six participants were needed per group to detect a smallest important change in means of 4.6 ng/ml (the increase required to elevate urinary NGAL above the normal range), assuming a within subject error of 1.7 ng/ml, and allowing for a 5 and 20% chance of making a Type I or Type II error, respectively (Hopkins 2006).

2.4. *RESULTS*

Descriptive data. All participants were Caucasian (figure 2.1.). Estimated GFR was similar in both groups (PeP positive: 121 ± 8; PeP negative: 115 ± 13 ml/min/1.73m^2; P = 0.3) and within healthy ranges for all participants. In the PeP positive group, mean age was slightly higher (PeP positive: 24 ± 5; PeP negative: 20 ± 2 yrs; P = 0.04) and there were more females (PeP positive: 5 females; PeP negative: 2 females).

As there were no differences between groups for hydration status and exercise intensity by independent *t*-tests, data from both groups were combined. Pre-800 metre run urine osmolality was 173 ± 119 mOsmols/kgH_20, indicating good hydration status prior to testing. There was the expected elevation of serum lactate between pre- and 0 hours post-run (1.2 ± 0.4 and 8.3 ± 3.1 mmol/l, respectively; P < 0.001), indicating physical exercise was completed at a high intensity.

Albumin:creatinine. Despite identification of PeP positive and PeP negative subjects during screening, the experimental protocol yielded a rise in A:Cr post-exercise in *all* participants who provided urine samples by 25 minutes. At this time point, mean A:Cr peaked at 27.6 {11.7, 50.5} mg/mmol before returning close to pre-exercise levels of 1.2 {0.9, 1.4} mg/mmol, by two hours with a significant effect of time (P < 0.001; *post-hoc* analyses, pre to 25 minutes: P = 0.001; pre to two hours: P = 0.4). There was a trend for a positive correlation between percentage change in serum lactate and percentage change in A:Cr (between pre and 25 minutes; r = 0.493; P = 0.07).

As all participants unexpectedly developed PeP at 25 minutes, a new A:Cr cut-off criteria was applied to examine the quantitative effects of proteinuria upon NGAL release. Therefore, the participants were divided into macroproteinuria (A:Cr > 30 mg/mmol; N = 6) and non-macroproteinuria (A:Cr < 30 mg/mmol; N = 8) groups by assessing their peak A:Cr value at 25 minutes (Poortmans 1984). Baseline estimated GFR and age were not significantly different between these two newly defined groups (data not shown). Consequently, and by design, for A:Cr there was a time × macroproteinuria status interaction (figure 2.2a). Examination of individual plots clearly showed that most peaks in A:Cr occurred at 25 minutes, but there were five individuals who peaked earlier immediately post-exercise (figure 2.2b).

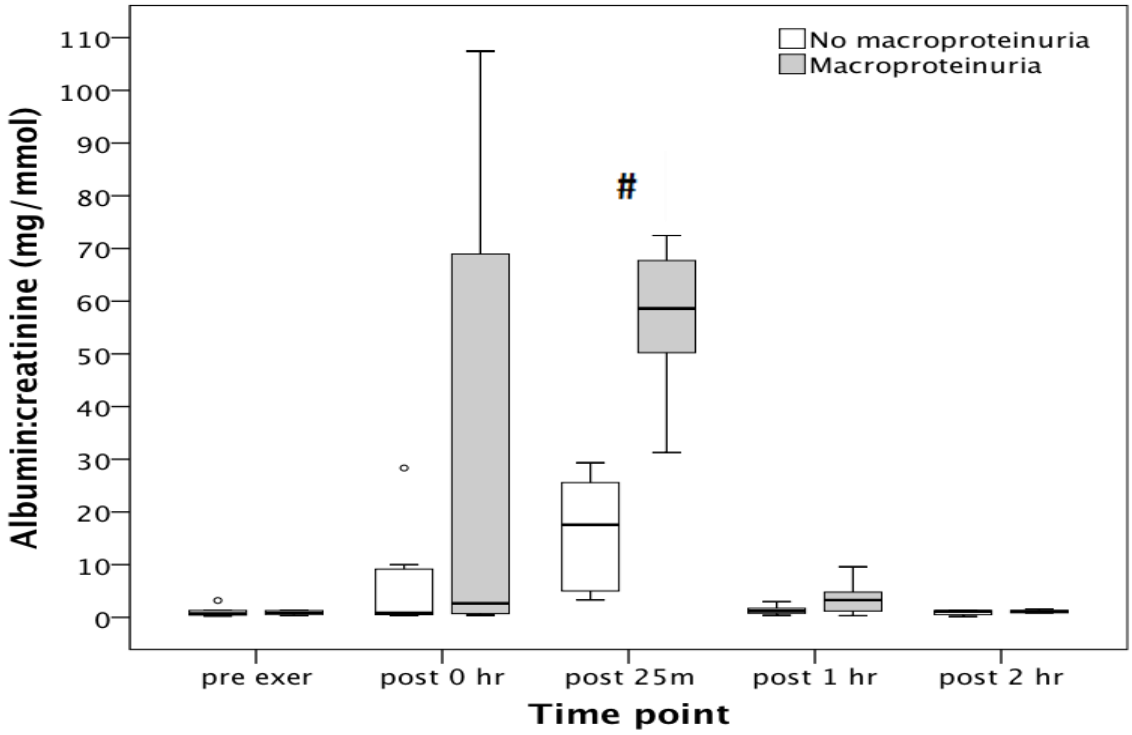

Figure 2.2a. Effect of an 800 metre run on albumin:creatinine ratio in non-macro and macroproteinuria subgroups. Data are medians (thick lines) and interquartile ranges (boxes). Open circles are outliers. There was a significant interaction between time and macroproteinuria status (P = 0.001 by Quade's test). #, difference between non-macro and macroproteinuria groups by *post hoc* test.

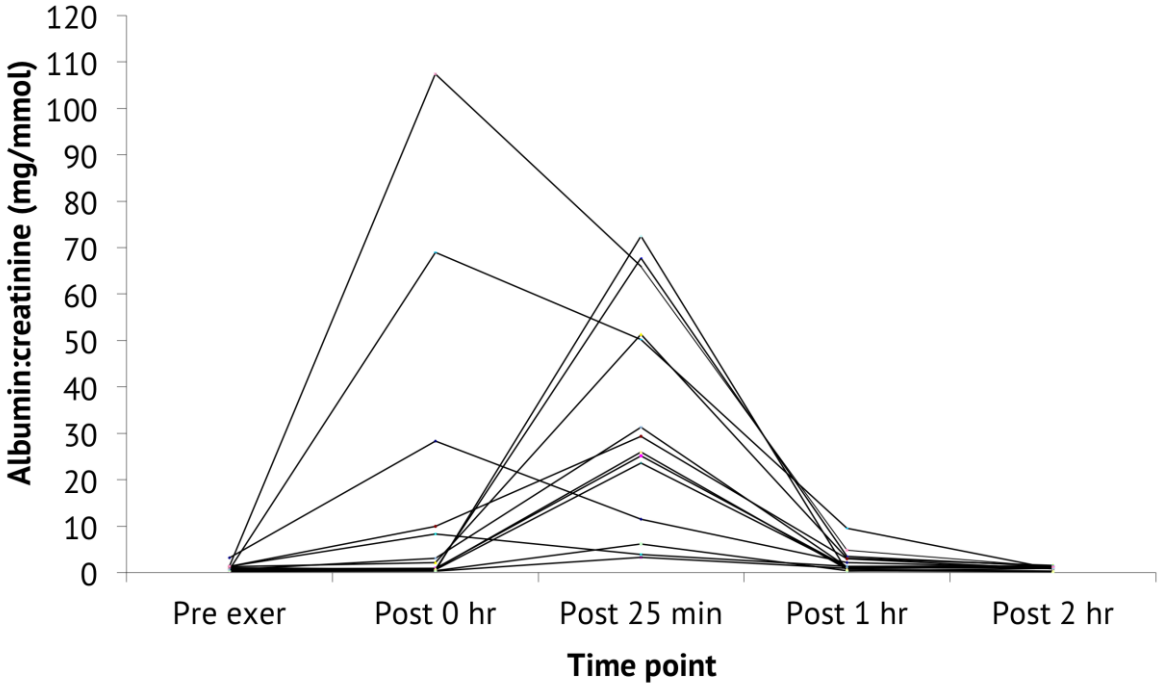

Figure 2.2b. Individual plots for albumin:creatinine ratio following an 800 metre run.

Urine volume. Only 14/20 participants (70%) were able to provide urine samples at all time points following the 800 metre run. Thus, subsequent reporting of results and statistics refers only to this cohort. This observation was accounted for by an exercise-induced anti-diuretic effect that was most pronounced at 25 minutes, when only 38 {28, 99} ml of urine could be produced. This oliguria recovered by two hours to 420 {204, 586} ml. Overall, there was a significant effect of time (P = 0.001; *post-hoc* analyses, pre to 0 hours: P = 0.004; pre to 25 minutes: P = 0.001; pre to two hours P = 0.5; figure 2.3). Response in urine volume was similar between macroproteinuria and non-macroproteinuria groups (no interaction effect: P = 0.5); therefore both groups are combined in figure 2.3.

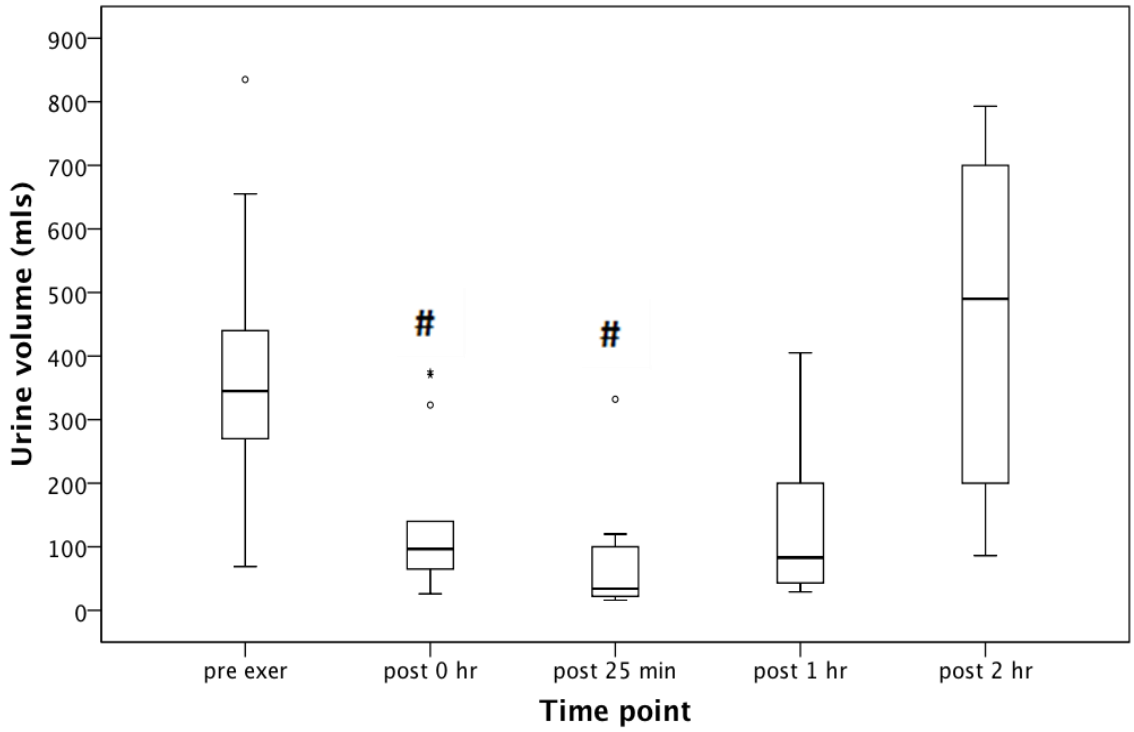

Figure 2.3. Effect of an 800 metre run on urine volume. Data are medians (thick lines) and ranges. Open circles and asterixes are outliers. There was a significant effect of time (P = 0.001 by Friedman's test). #, difference compared to pre-exercise sample point by *post hoc* test.

Urinary NGAL. There were increases in urinary NGAL following exercise which peaked at 12.8 {6.2, 23.2} ng/ml at 25 minutes before decreasing below baseline levels to 0.8 {0.02, 3.3} ng/ml at two hours (significant effect of time: P = 0.002; *post-hoc* analyses, pre to 25 minutes: P = 0.04 (not significant after Bonferroni correction); pre to two hours post: P = 0.004; figure 2.4a). At 25 minutes and two hours, urinary NGAL was negatively correlated with percentage change in urine volume (pre to 25 minutes; ρ = -0.701; P = 0.005; 25 minutes to two hours; ρ = -0.552; P = 0.04). Response in urinary NGAL was similar between

macroproteinuria and non-macroproteinuria groups (no interaction effect; $P = 0.6$); therefore both groups are combined in figure 2.4a. Individual data plots suggested a cluster of urinary NGAL peaks occurring at 0 and 25 minutes post although there was marked inter-individual variation (figure 2.4b). Throughout the entire post-exercise period, nine individuals (64%) showed an elevation above the normal range at one or more time points, with one subject attaining a concentration of 145 ng/ml (figure 2.4b).

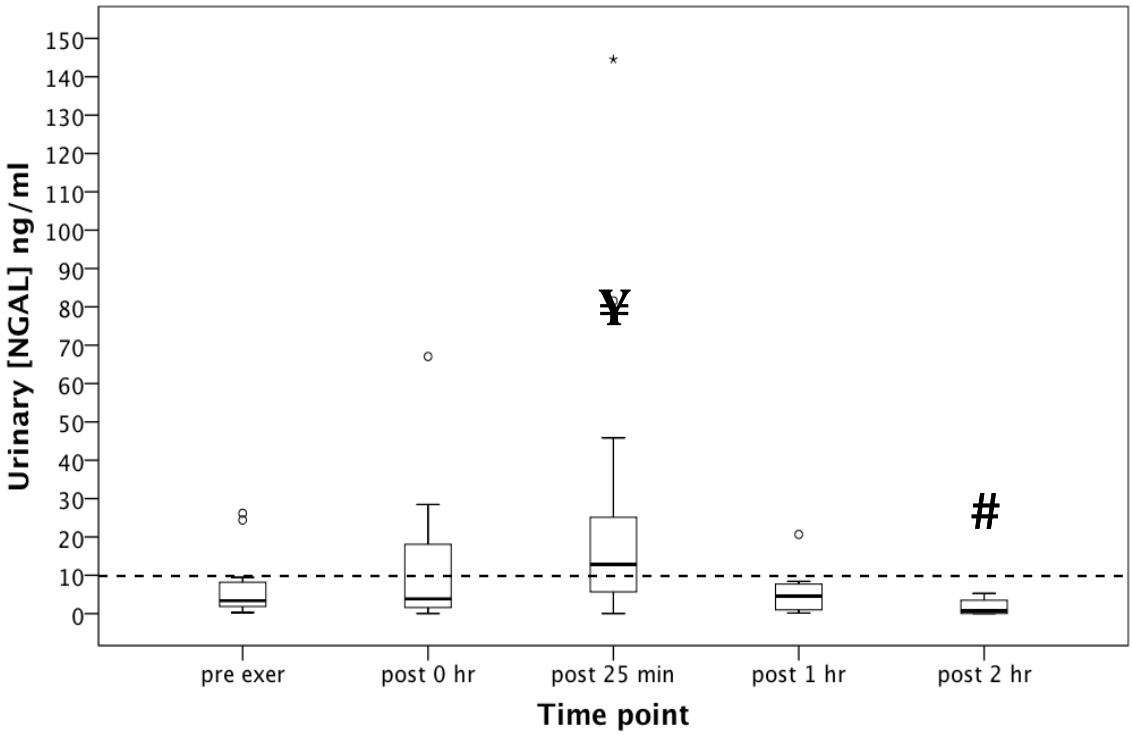

Figure 2.4a. Effect of an 800 metre run on urinary NGAL concentration. Data are medians (thick lines) and interquartile ranges (boxes). Open circles and asterixes are outliers. Dotted line indicates upper limit of normal range for urinary NGAL. There was a significant effect of time ($P = 0.002$ by Friedman's test). #, difference compared to pre-exercise sample point by *post hoc* test; ¥, difference compared to pre-exercise sample point by *post hoc* test but not significant after Bonferroni correction.

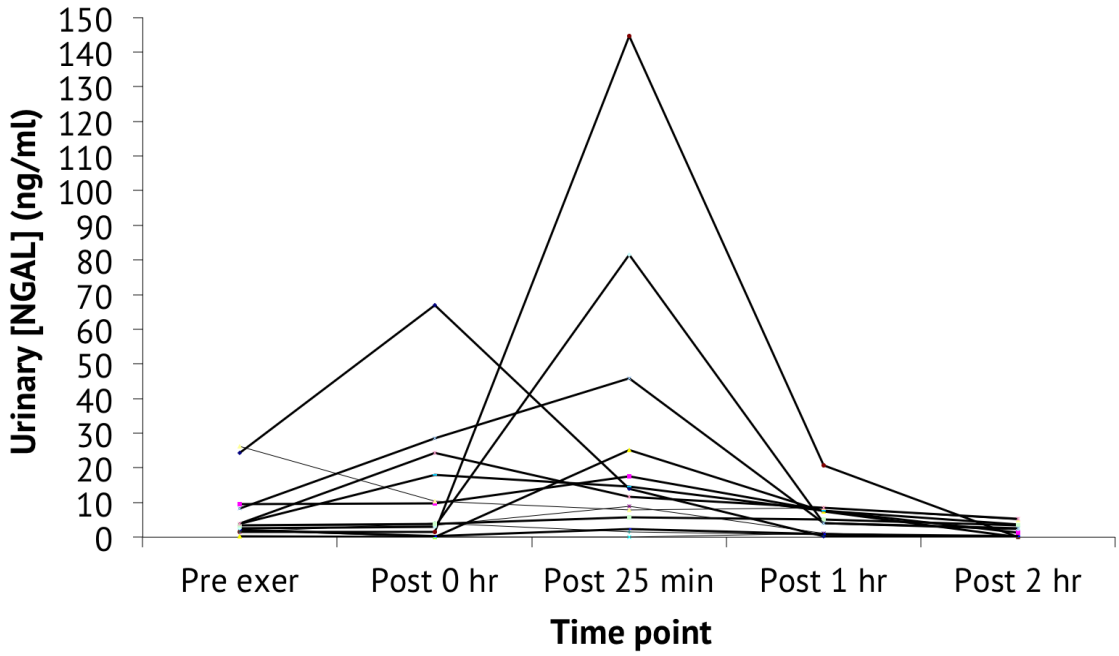

Figure 2.4b. Individual plots for urinary NGAL concentration following an 800 metre run.

Urinary NGAL:creatinine. Although median urinary NGAL:Cr appeared to be relatively stable at pre, 0 hours and 25 minutes, it fell significantly at two hours to $0.42x10^{-3}$ {$0.02x10^{-3}$, $0.1x10^{-3}$} ng/mmol with a significant effect of time (P = 0.002; *post-hoc* analyses, pre to two hours: P = 0.004; figure 2.5a). Response of urinary NGAL:Cr was similar between macro- and non-macroproteinuria groups (no interaction effect; P = 0.241); therefore both groups are combined in figure 2.5a. When individual data were inspected, there were clusters of urinary NGAL:Cr peaks occurring at 0 and 25 minutes although there was marked inter-individual variation (figure 2.5b).

Plasma NGAL. Baseline plasma NGAL was 73.0 ± 17.0 ng/ml and there was a significant effect of time (P = 0.002). However this was due to a *decrease* in plasma NGAL concentration from baseline at 25 minutes to 68.8 ± 15.0 ng/ml and at one hour to 64.5 ± 16.7 ng/ml (*post-hoc* analyses, pre and 25 minutes: P < 0.001; pre and one hour: P = 0.007; pre and two hours: P = 0.03 (not significant after Bonferroni correction); figure 2.6). Similar to urinary NGAL and urinary NGAL:Cr, there was no interaction with type of proteinuria for plasma NGAL (P = 0.7). Throughout the entire post-exercise period, no individual went above or below the normal range for plasma NGAL at any time.

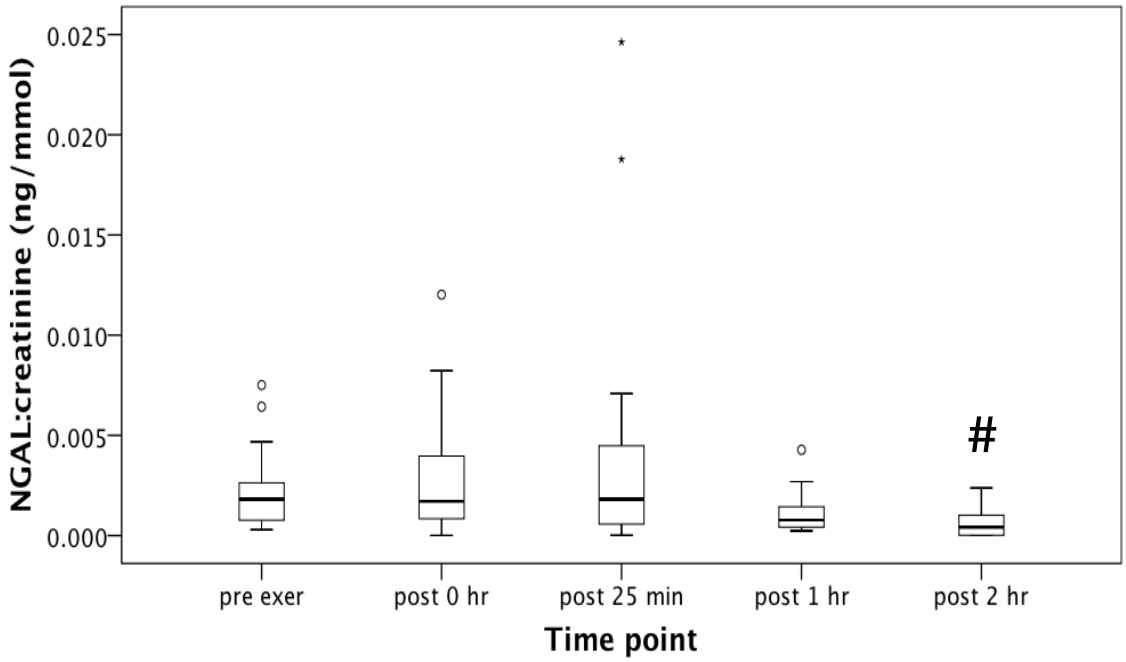

Figure 2.5a. Effect of an 800 metre run upon urinary NGAL:creatinine ratio. Data are medians (thick lines) and interquartile ranges (boxes). Open circles and asterixes are outliers. There was a significant effect of time (P = 0.002). #, difference compared to pre-exercise sample point by *post hoc* test.

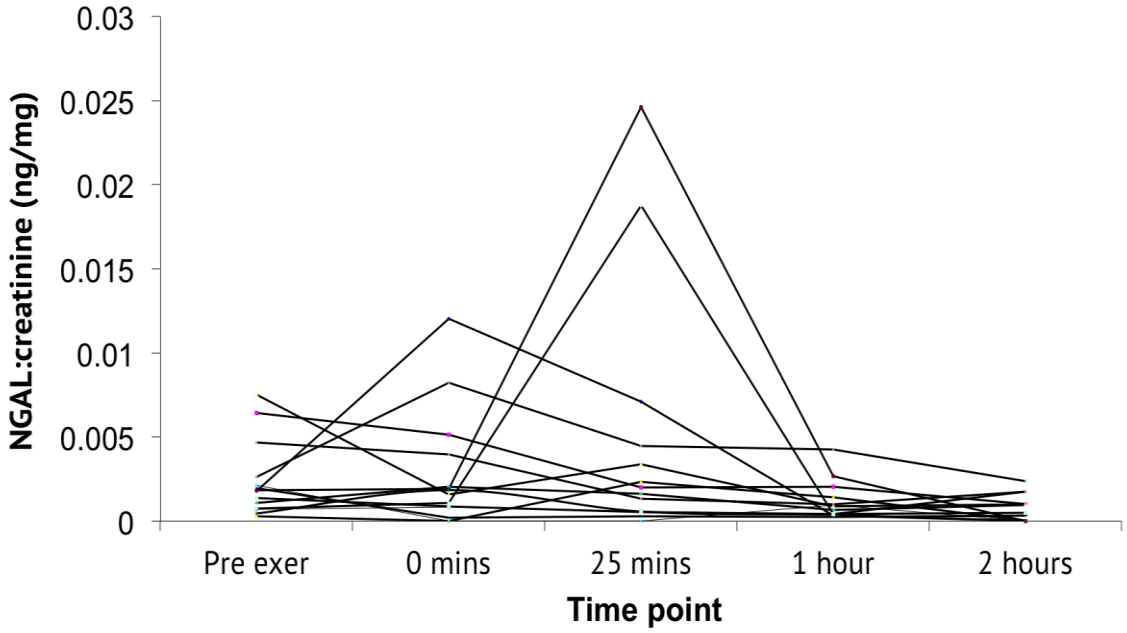

Figure 2.5b. Individual plots for urinary NGAL:creatinine ratio following an 800 metre run.

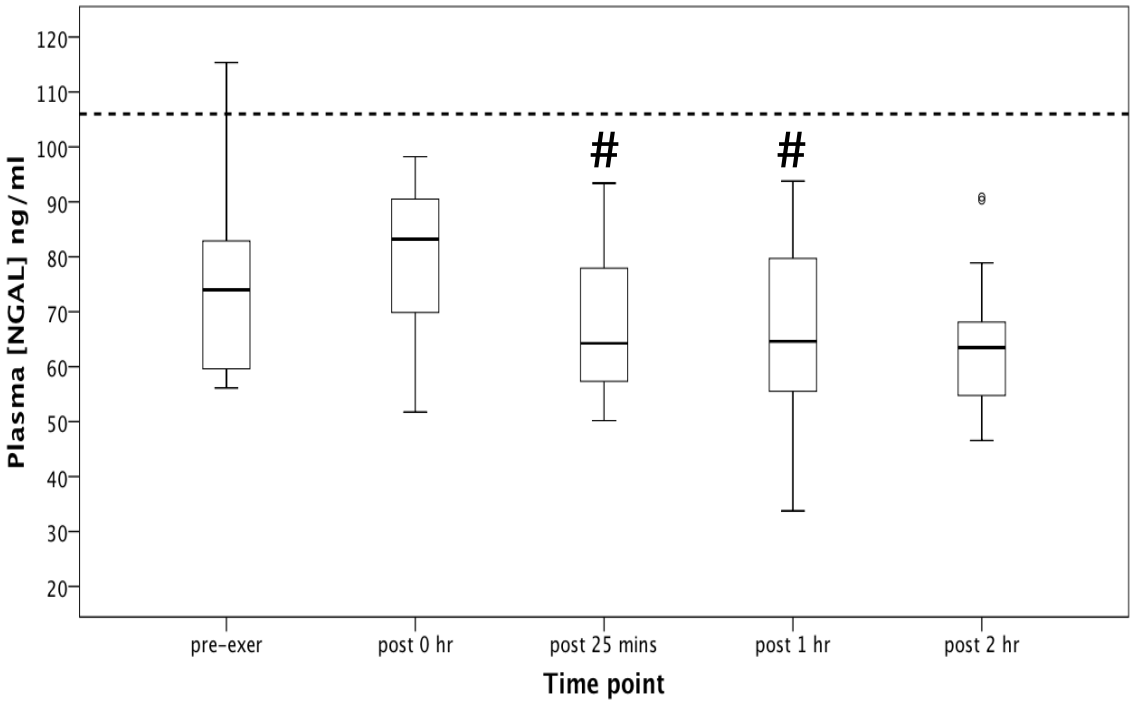

Figure 2.6. Effect of 800 metre run upon plasma NGAL level over time. For the sake of consistency, data are presented as boxplots whereas data in results section are mean (±SD). Data are medians (thick lines) and interquartile ranges (boxes). Open circles are outliers. Dotted line indicates upper limit of normal range for plasma NGAL. There was a significant effect of time ($P < 0.002$). #, difference compared to pre-exercise sample point by *post hoc* test.

2.5. *DISCUSSION*

This is the first study to examine NGAL kinetics and its association with proteinuria following short duration high-intensity exercise. These data partly supports upregulation of NGAL with a trend for a transient elevation in urinary NGAL concentrations following an acute bout of high-intensity exercise (pre to 25 minutes, $P = 0.04$; not significant after Bonferroni correction). However, this response was not uniform and demonstrated large inter-individual variation. Furthermore, there was a negative correlation between urinary NGAL and percent change in urine volume, and median increases in urinary NGAL were attenuated when expressed relative to urinary Cr. Taken together these data suggest that elevations in urinary NGAL following exercise may in part be due to a concentration effect. In addition, statistically significant decreases in plasma NGAL only occurred 2 hours post-exercise (figure 2.5b.).

The current data do not support the hypothesized association between NGAL response and the extent of post-exercise proteinuria. Nevertheless, these data provide important information on the site-specific renal stress experienced during high-intensity exercise, and have implications for interpretation of NGAL within the clinical environment.

Although the functions of NGAL have yet to be fully elucidated, there is an early and dramatic upregulation of NGAL in AKI (Allen *et al.*, 1989; Goetz *et al.*, 2002; Mishra *et al.*, 2003; Mishra *et al.*, 2005). More than half of participants in the current study had urinary NGAL elevations above the normal range at 25 minutes post-exercise, but unsurprisingly none of the participants reached the currently used cut-off diagnostic for AKI (250ng/ml) (NGAL Rapid ELISA kit, Bioporto, Gentofte, Denmark). However, this cut-off has been derived from a diseased population and whether more modest elevations indicate a milder form of kidney stress or injury has not been investigated.

At the time of writing, there was only one published study which reported the effect of exercise upon NGAL kinetics. McCullough *et al.* measured urinary NGAL in 25 individuals during and after a marathon (McCullough *et al.*, 2011). These subjects demonstrated a significant mean elevation, from 8 ± 4 ng/ml pre-marathon to 47 ± 29 ng/ml immediately post marathon ($P < 0.05$). The peak median urinary NGAL value observed in our investigation was considerably lower at 13 (interquartile range 17) ng/ml. Thus, if group averages are compared, prolonged exercise likely results in greater NGAL release compared to short-duration higher-intensity activity. However, similar to the current findings, McCullough *et al.*

also noted wide variation for peak urinary NGAL with values in some individuals above 100 ng/ml.

Such urinary NGAL elevations could be a result of increased inflammatory injury to nephron units, specifically at the proximal tubule, with subsequent impairment in function (Poortman *et al.*, 1988; Mishra *et al.*, 2004; Mishra *et al.*, 2005; Mishra *et al.*, 2006). This may affect tubular reabsorption of NGAL as reabsorption of other small molecular weight proteins, such as lysozyme and β_2 – microglobulin, are known to be inhibited by exercise (Kozlowski *et al.*, 1967; Poortmans 1984; Poortmans *et al.*, 1997). Leucocytouria has also been noted as a source of urinary NGAL elevations in the absence of AKI (e.g. in urinary tract infection) and analysis of urinary sediments following exercise has demonstrated that long duration activity is more likely to result in an increased leucocytouria (and haematuria), albeit this finding is not universal (Poortmans 1984; Decavele *et al.*, 2011). Detection of dimeric urinary NGAL isoforms from neutrophils may distinguish release of urinary NGAL from tubular epithelium, as these are predominantly monomeric (Mårtensson *et al.*, 2012). In the present study, urine samples for leukocyte quantification were not collected and the ELISA kit utilized in this study only detected monomeric urinary NGAL. Whilst the possible contribution from this source merits further investigation, the urinary data suggests that even short exercise bouts induce a mild kidney stress.

The present findings also highlight the difficulties of interpreting urinary biomarkers. Currently, there is no clear agreement on how renal biomarkers values from urine should be expressed and the assay kit utilized in this study bases interpretation of urinary results on raw values which fail to consider specific correction factors (Waikar *et al.*, 2010). For example, the elevation in median urinary NGAL at 25 minutes may in part be explained by the oliguria at that same time point, a conclusion supported by the significant negative correlation with percentage change in urine volume between pre and 25 minutes. Exercise is known to exert an anti-diuretic effect with numerous studies demonstrating a decrease in free water clearance in both dehydrated and hyperhydrated states (Kozlowski *et al.*, 1967; Poortmans 1984). It is also plausible that the significant decrease in median urinary NGAL at two hours is due to relative polyuria following a period of anti-diuresis; this is supported by correlation with percentage change in urine volume. This situation is analogous to the shortcomings of measuring AKI biomarkers in urine during oliguric AKI (Waikar *et al.*, 2010). Therefore, in an attempt to circumvent the issues surrounding changes in urinary concentration, urinary NGAL concentrations were normalized to urinary Cr. Although this correction resulted in the dissipation of median NGAL rises and correlations with changes in urinary volume, it is

noteworthy that some individuals continued to show substantial rises in urinary NGAL:Cr (particularly at 25 minutes).

As Cr generation is not constant during exercise, normalisation of urinary NGAL to urinary Cr could also be considered inappropriate. However, it is interesting to note that previous studies have utilised A:Cr to demonstrate post-exercise proteinuria. Instead, observed urinary NGAL concentrations could be normalised to urinary flow rate, hence permitting determination of a biomarker excretion rate (Waikar *et al.*, 2010). Unfortunately flow rates could not be calculated in the present study. As the first urine collection was done at pre-exercise and time the subject had micturated prior was not recorded, a baseline urinary NGAL flow rate could not be calculated. Of course, flow rates could be calculated subsequent to this but these would be uninterpretable without a baseline. There has also been suggestion that urine osmolality and conductivity could be used as an alternative normalization measures (Tomonaga *et al.*, 2012). Assuming a steady state, it could be useful. However, in short-term acute exercise of moderate intensity there is an increase in urine osmolality of around 40 mOsmols/KgH_20 (Freund *et al.*, 1991). In addition, high-intensity exercise as in the study's protocol may result in further decreases in tubular function. Thus, it is not certain that in a dynamic state such as exercise (or indeed AKI), these normalisation techniques would be helpful. Nevertheless, as normalisation of urinary NGAL remains problematic, this is an area that merits further investigation. Overall, the current data suggest that elevations in urinary NGAL should be interpreted with caution in both research and clinical settings, as elevated NGAL may in part be due to urinary concentration.

Measurement of plasma NGAL largely avoids the confounding factors affecting interpretation of its urinary equivalent. After correcting for plasma volume shifts, the current study showed no significant elevation in plasma NGAL. In contrary there was a minor yet statistically significant *decline* in plasma NGAL from 25 minutes that coincided with the peak urinary NGAL concentration. Thus, it is plausible that urinary NGAL elevations may arise from the plasma due to increased clearance into the Bowman's capsule via the glomerular capillaries. This may involve exercise-related reductions in renal blood flow to catecholamine surges with corresponding falls in GFR and increases in filtration fraction (Kadachorian and Johnson, 1970; Poortmans 1984).

The present study did not demonstrate any relationship between NGAL and the degree of PeP, nor between NGAL and any descriptive characteristics. Thus participants with post-exercise macro-proteinuria reassuringly appear to be no more susceptible to exercise-induced kidney stress or injury than those without macro-proteinuria. Admittedly, with the small

sample size a type II statistical error is possible. However, the study was powered to detect a minimally important clinical change (NGAL elevations above the normal range). Additionally, distinct mechanisms seem to be responsible for post-exercise macro-proteinuria and NGAL up-regulation in nephron units. It is of note that 100% of individuals tested in the current study presented with PeP (as initially defined) following maximal exercise, supporting previous findings that when exercise is of sufficient intensity, all individuals will have some degree of PeP (Kozlowski *et al.*, 1967; Poortmans *et al.*, 1997).

Limitations of this study include a lack of bladder ultrasound or urinary catherization. Although double voiding was encouraged, gold standard measurements of urine volume and therefore urine flow were not made, meaning biomarker production rate could not be calculated. To mitigate this limitation, participants arrived well hydrated, urinary data were presented relative to Cr concentration, and NGAL was also obtained as a plasma concentration, expressed relative to plasma volume shifts. Study strengths include the use of a "real-world" exercise protocol and selection of non-elite athletes; both of which support the applicability of these results to a young active healthy population. The repeated measures design and the conservative approach to statistical analyses, using non-parametric analyses on data which is characteristically skewed, is also well-suited to the study's small cohort.

In summary, for the first time it has been shown that an acute bout of high intensity exercise results in transient urinary NGAL elevations that are independent of proteinuria. Sources of NGAL may include the proximal tubule but also increased plasma clearance. However, absolute urinary values need to be interpreted with caution due to the anti-diuretic state exerted by exercise. Normalizing urinary NGAL to urinary Cr appeared to attenuate absolute rises. Given such phenomena, further work needs to be undertaken to determine the most suitable means of NGAL expression, e.g. biomarker excretion rate. Finally, given the large inter-individual response to urinary NGAL up-regulation, more research is needed to determine the factors responsible for large increases in some individuals and their clinical implications. These endeavours are essential if NGAL and other promising renal biomarkers are to be validated as measures of renal injury following exercise or oliguric AKI and to avoid erroneous interpretation.

ACKNOWLEDGEMENTS

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CHAPTER 3

CAN EXERCISE-INDUCED PROTEINURIA PREDICT THE ONSET OR PROGRESSION OF CHRONIC KIDNEY DISEASE IN HUMANS? A SYSTEMATIC REVIEW

3.1. *ABSTRACT*

Background: Proteinuria is a biomarker of chronic kidney disease (CKD). Post-exercise proteinuria (PeP) shares certain characteristics with proteinuria of CKD including generation through increased intraglomerular pressures, a predilection for albumin excretion and reductions in glomerular filtration rate. Objective: To determine if PeP can predict the onset or progression of CKD. Data sources: A systematic review of English, human subject-based articles published between 1946 and 2014 in Ovid Medline (R), Ovid Medline in process, AMED, EMBASE, Pubmed, Cochrane Library and Web of Science. Search terms included exercise, exercise test, proteinuria, albuminuria, post-exercise proteinuria, post-exercise albuminuria, exercise induced-proteinuria and exercise induced-albuminuria, chronic kidney disease and nephropathy. Study eligibility criteria: Participants were individuals with or at-risk of CKD. Intervention was an exercise test designed to yield PeP. The comparator population were individuals who did not develop PeP after exercise testing. Outcomes included biomarkers of CKD (e.g. high resting proteinuria or rise in blood creatinine) from a follow-up period of at least three months. Study types examined: randomized controlled trials and prospective observational cohort studies. Study appraisal and synthesis methods: Independent extraction of articles and data items by two authors using predefined data fields including study quality indicators. Discrepancies identified were resolved through a co-investigator. Risk of bias and applicability was asessesed by the QUADAS-2 tool. Results: A narrative synthesis was performed. Five studies (N = 351) met inclusion criteria, all of which examined prospective cohorts of Type I diabetics who were at risk of CKD. Meta-analysis was not possible as interventions and post-exercise measurements varied markedly between studies. However, when combining the results of the primary outcome in four studies (N = 318), the presence of PeP was highly associated with elevated resting proteinuria at follow-up (χ^2 test, $P < 0.0001$) and significant odds ratios (developing CKD following a positive exercise test vs. not developing CKD) were noted for each of four studies (OR 2.3-52). Limitations: findings are only applicable to a population of young type I diabetics at risk of CKD. Interventions were a source of bias with variance in exercise intensity and duration between studies. Primary outcomes did not factor confounding variables such as use of angiotensin-receptor antagonists and the development of other nephropathies. Conclusion and implications: Despite the limited number of studies in the literature and their shortcomings, PeP shows promise as a predictor for CKD progression. However, there is a need to refine exercise testing for this purpose. PROSPERO registration number: CRD42014008686.

3.2. *INTRODUCTION*

Through experimental study of chronic kidney disease (CKD), long-standing proteinuria resulting from glomerular hypertension is an independent predictor for CKD progression (Abbate *et al.*, 2006). At a cellular level, podocytes exposed to an excessive protein load release transforming growth factor beta, ultimately inducing differentiation of mesangial cells into myofibroblasts (Yard *et al.*, 2001; Abbate *et al.*, 2002). Further along the nephron, protein overload in the tubules induces the release of cytokines, chemokines and growth factors that lead to abnormal interstitial accumulation of inflammatory cells, extracellular matrix collagen, fibronectin and other components that are responsible for interstitial fibrosis; a histopathological hallmark of CKD (Kriz and LeHir 2005; Abbate *et al.*, 2006). Typically through the use of renin-angiotensin-aldosterone system inhibitors that lower blood pressure and intraglomerular pressures, proteinuria and its adverse effects can be halted (Brenner *et al.*, 2001). Thus, the detection and treatment of proteinuria asserts great importance in CKD management strategies (Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group, 2013).

In contrast, proteinuria in the context of exercise is a well-recognised but transient physiological phenomenon that is currently thought to be benign, even when encountered on repeated occasions (Poortmans 1985; Poortmans and Vanderstraeten, 1994). However, convincing evidence for this is lacking, although long-term data on other so-called "physiological proteinurias" (e.g. orthostatic proteinuria) does not demonstrate any detrimental effects upon renal function (Glassock 1981; Springberg *et al.*, 1982). Nonetheless, both proteinuria resulting from CKD and post-exercise proteinuria (PeP) share certain characteristics including potential mechanisms of generation such as increased intraglomerular pressures, a predilection for albumin excretion, and reductions in glomerular filtration rate (GFR; Refsum and Strömme, 1975; Poortmans 1984, Bellinghieri *et al.*, 2008).

Given the above similarities, the notion of whether proteinuria provocation through physical exertion could be used as a kidney stress test to predict the onset or prognosis of nephropathies is of particular interest. Exercise stress tests are already utilised for cardiac disease risk stratification e.g. through use of the Bruce protocol (Lauer *et al.*, 2005; Bourdillon 2010). Physical exertion as a kidney stress test potentially fulfils a number of desirable characteristics for a useful disease biomarker, including: ease of performing investigation (treadmill exercise protocols are routinely completed in cardiac patients and established proteinuria assays are readily available), ease of obtaining sample (spot urine

collection), rapid throughput and result generation (due to routine and commonly available biochemistry); and ability to offer treatment (anti-proteinuric agents).

Already, there is evidence that analysis of the urinary proteome could offer important prognostic information about specific nephropathies (Mullen *et al.*, 2011; Santucci *et al.*, 2013). However, there is presently no consensus on whether PeP could be a useful diagnostic tool to predict the onset or progression of CKD. This is an important question as such a test would provide a useful means of diagnosing the onset of nephropathies at various stages and may provide an adjunctive or triage assessment before more risky invasive procedures such as renal biopsy. Additionally, it could allow for early proactive measures to prevent disease progression. Therefore, the aim of this study was to perform a systematic review and assess all existing literature to determine if proteinuria provocation following exercise testing can predict onset or progression of CKD in humans.

3.3. *MATERIAL AND METHODS*

Study registration. The study was registered under PROSPERO – the international prospective register of systematic reviews. For full online entry, the reader is directed to: http://www.crd.york.ac.uk/PROSPERO/display_record.asp?ID=CRD42014008686.*Eligibility criteria.* The research question was formulated into the **PICOS** format. **P**articipants: this comprised of three eligible groups: 1) individuals with CKD; defined as abnormalities of kidney structure and function present for no less than three months (table 3.1.); 2) those with diseases that confer a risk of CKD e.g. diabetics; or 3) healthy individuals with a family history of inherited renal disease in first-degree relatives e.g. polycystic kidney disease. **I**ntervention: an exercise tolerance test or similar bout of exercise likely to yield PeP (Poortmans 1984; Junglee *et al.* 2012). **C**omparator: participants listed above that did not develop PeP as per study limits following an exercise tolerance test or similar bout of exercise. **O**utcomes: primary outcomes were indicators of CKD progression after a follow-up period of at least three months including: 1) increased blood creatinine (Cr) concentration of 25% from baseline; 2) a drop in estimated GFR category accompanied by a 25% or greater drop in estimated GFR from baseline (table 3.2.); or 3) resting proteinuria greater than 3 mg/mmol albumin:creatinine ratio (A:Cr), 30 mg/g protein:creatinine ratio (P:Cr), 20 ug/min albumin excretion rate or other contempory measures of pathological resting proteinuria as recognized by a consensus group (Junglee *et al.*, 2012; Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group, 2013). Secondary outcomes (i.e. not a prerequisite for inclusion in the systematic review but were reported if studied), included any biomarkers associated with CKD progression e.g. worsening hypertension (blood pressure > 140/90 mmHg) or glycaemic control (glycosylated haemoglobin, HbA1c) in diabetics. **S**tudy type: randomized controlled trials and prospective observational cohort studies.

Markers of kidney damage (one or more)	Albuminuria (albumin excretion rate >30 mg/24 hours; A:Cr >30 mg/g [>3 mg/mmol])
	Urine sediment abnormalities
	Electrolyte and other abnormalities due to tubular disorders
	Abnormalities detected by renal biopsy
	Structural abnormalities detected by imaging
	History of kidney transplantation
Decreased GFR	GFR <60 ml/min/1.73 m^2

Table 3.1. Criteria for definition of CKD based upon the KDIGO Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease 2012. Either of the key features (left-hand side in bold) need to be satisfied for a period of no less than three months to fulfil a diagnosis of CKD. A:Cr, albumin creatinine ratio; GFR, glomerular filtration rate. Adapted from Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group, 2013.

GFR category	GFR (ml/min/1.73m^2)
1	≥ 90
2	60-89
3a	45-59
3b	30-44
4	15-29
5	< 15

Table 3.2. Stage of chronic kidney disease as defined by the KDIGO Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease 2012. GFR, glomerular filtration rate. Adapted from Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group, 2013.

Thus, primary studies, published in English and containing human participants of any age, sex, or ethnicity were sought. Animal studies, *in-vitro* studies and those not written in English language were rejected as data only relevant to exercise testing as applied to humans was required and there would be economical and time constraints in translating studies written in other languages.

Information sources. With the assistance of a medical librarian, a search of the following electronic databases restricted to publications from 1946 to November 2014, was performed: Ovid Medline (R), Ovid Medline in process, AMED, EMBASE, Pubmed, Cochrane Library

and Web of Science. The search was performed on the 7th of November 2014. Relevant review, case reports and commentaries were hand-searched for other potentially relevant references. Reference lists of all selected papers were also hand-searched for other relevant references missed from electronic database enquiries. Also, where appropriate, main authors of studies meeting eligibility criteria were contacted to identify additional studies. Grey literature was not searched.

Search strategy. Using OvidSP (https://ovidsp.uk.ovid.com), electronic databases including Ovid Medline (R), AMED and EMBASE were searched using the following Medical Sub-Heading terms separately and / or in combination: exercise, exercise test, proteinuria, albuminuria, chronic kidney disease and nephropathy (for example of search enquiries see appendix 1). Non-MESH terms including: post-exercise proteinuria, post-exercise albuminuria, exercise induced-proteinuria and exercise induced-albuminuria were also searched for separately. Limits applied included studies involving humans and in the English language. For each database, the search strategy had to be adjusted to ensure the optimum number of hits. A similar process was conducted for: Pubmed (http://www.ncbi.nlm.nih.gov/pubmed); Cochrane Library (http://onlinelibrary.wiley.com/cochranelibrary/search); and Web of Science (http://apps.webofknowledge.com).

Study selection. Following the removal of duplicate entries from the database searches, two review authors (Naushad Ali Junglee & Jamie Hugo Macdonald), using pre-defined criteria, independently evaluated each potential study through evaluation of the title and abstract (figure 3.1.). Reviewers compared their assessments and any disagreements were resolved by discussion with a co-investigator (Andrew Bruce Lemmey).

Data collection process. A standardized, pre-piloted form was used to extract data from the selected studies for assessment of study evidence and quality synthesis. The two review authors extracted data independently. Any discrepancies identified were resolved through discussion with a co-investigator and attempts were made to retrieve missing data from study authors.

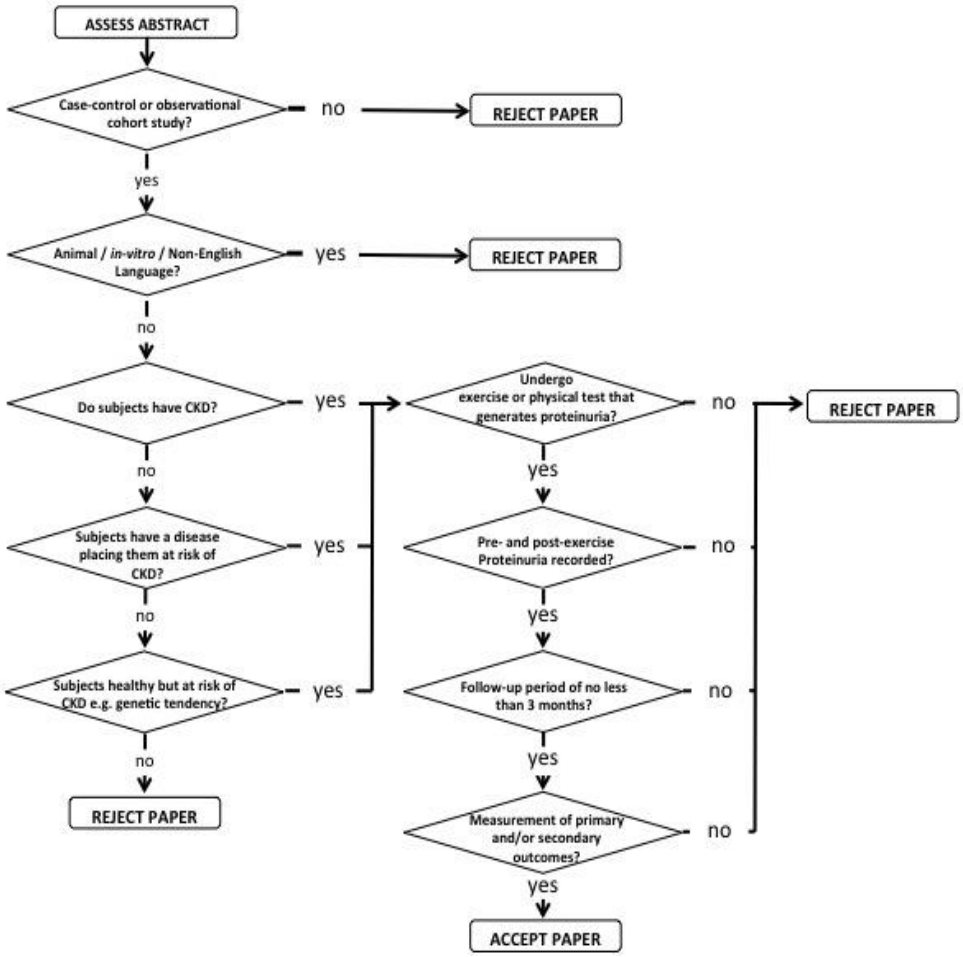

Figure 3.1. Flowchart used to select papers into systematic review. Specific values for definition of post-exercise proteinuria and primary outcomes are detailed in the *Eligibility Criteria* section. Inclusion of secondary outcomes were not an essential prerequisite for study inclusion. CKD: chronic kidney disease.

Data items. Extracted information included: study design; type of nephropathy examined; study population characteristics; comparator population characteristics; exercise intervention employed; definition of exercise proteinuria; pre- and post-exercise proteinuria concentrations; follow-up time period; primary outcomes and any association with post-exercise proteinuria; secondary outcomes and any associations with post-exercise proteinuria; and information for assessment of the risk of bias.

Risk of bias assessment of individual studies and across studies. For each selected study, risk of bias and quality assessment was evaluated independently by both review authors through application of the QUADAS2 tool (see appendix 2; http://www.bris.ac.uk/quadas/quadas-2/), which has been validated for primary diagnostic accuracy studies. This assessment focuses upon four key domains: 1. patient selection; 2.

index test; 3. reference standard; and 4. flow and timing. Each domain incorporated signaling questions with additional anchoring statements specific to the review question and permits the reviewer to make an overall assessment of bias (appendix 2 and 3). The proportion of studies with differing degrees of bias and applicability was also determined to reflect the risk of bias *across* studies. Bias was not used as a study exclusion tool.

Summary measures. From each selected study, the principle summary measures for the population under study and the comparator were collated in a tabular format.

Synthesis of results. It was anticipated that there would be limited scope for meta-analysis because of the great variation in the interventions used across the small number of existing trials. Therefore, a narrative synthesis of the findings from the included studies was conducted. This was structured around the type of CKD investigated, populations selected, chosen exercise test to induce proteinuria, follow-up period and primary / secondary outcome measures. In addition, risk ratios were calculated.

Additional analyses. No additional analyses were performed.

3.4. *RESULTS*

Study selection. Of 69 potential studies identified in electronic searches, only 5 studies (N = 351) met the inclusion criteria as outlined in figure 3.1. Figure 3.2. details the results of the search process with justifications at each review stage for study exclusion.

Study characteristics. The five studies that met inclusion criteria were: Bognetti *et al.* (1994), Dash and Torffvit (2003), Garg *et al.* (1990), O'Brien *et al.* (1995) and Watts *et al.* (1989). The data extracted for each study are summarised in table 3.3.

Risk of bias assessment of individual studies and across studies. Each study underwent a QUADAS-2 assessment – the results of which were used to generate summary charts to determine the risk of bias and concerns regarding applicability across studies (appendix 2 and 3; table 3.4, figure 3.3).

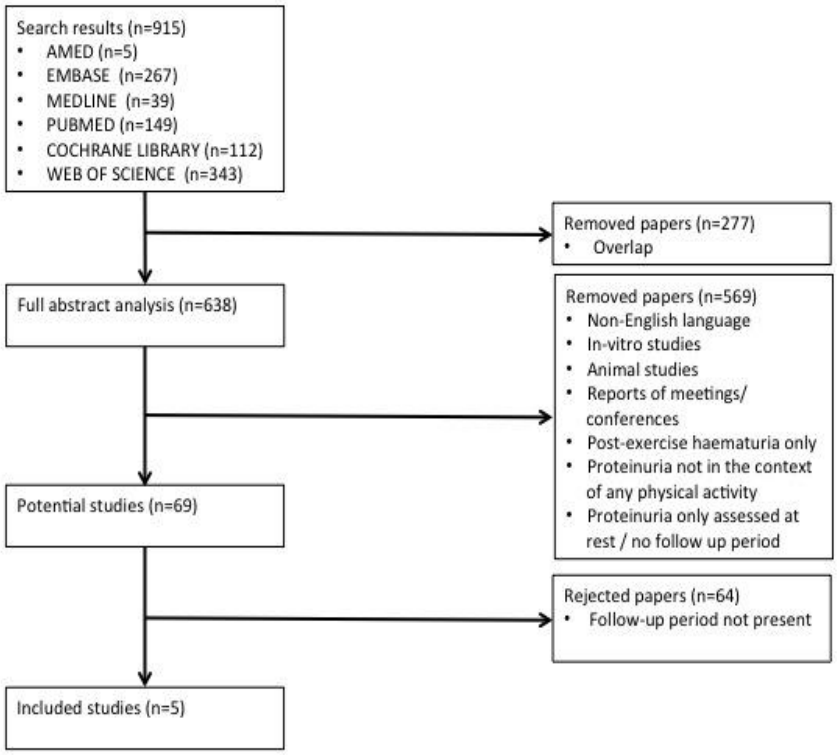

Figure 3.2. Flowchart detailing results of electronic database searches with reasons for exclusion at each stage.

	Bognetti *et al.* (1994)	Dash and Torffvit (2003)	Garg *et al.* (1990) [e]	O'Brien *et al.* (1995)	Watts *et al.* (1989)
Study type	**Prospective**	**Prospective**	**Prospective**	**Prospective**	**Prospective**
Nephropathy	**Type 1 diabetes**	**Type 1 diabetes**	**Type 1 diabetes**	**Type 1 diabetes**	**Type 1 diabetes**
Number of subjects	**66** [a]	**No microalbuminuria: 17** [b] **Microalbuminuria: 16**	**Group 1: 102** **Group 2: 20** **Group 3: 9** **Group 4: 20** **Group 5: 23** **Group 6: 9** **Group 7: 4**	**32**	**33**
Mean ± sd or median {range} age in years	**15.3 ± 3.1**	**No microalbuminuria: 27.0 ± 6.0** **Microalbuminuria: 30.0 ± 7.0**	**Group 1: 20.3 ± 0.3** **Group 2: 21.3 ± 0.6** **Group 3: 21.0 ± 0.6** **Group 4: 20.0 ± 0.6** **Group 5: 21.6 ± 0.6** **Group 6: 18.7 ± 0.4** **Group 7: 19.5 ± 1.2**	**28.7 ± 9.8**	**29.0 {17.0-49.0}**
Male:female ratio (N)	**40:26**	**No microalbuminuria: 17:0** **Microalbuminuria: 16:0**	**Group 1: 49:53** **Group 2: 10:10** **Group 3: 4:5** **Group 4: 9:11** **Group 5: 12:11** **Group 6: 7:2** **Group 7:4**	**25:7**	**26:7**
Mean ± sd or median {range} body mass index kg/m^2	**NS**	**NS**	**NS**	**24 ± 2.8**	**24.2 {17.1-27.6}**
Mean ± sd or median {range} duration of diabetes	**8.9 ± 3.0**	**No microalbuminuria: 13.0 ± 4.0** **Microalbuminuria: 14.0 ± 4.0**	**Group 1: 11.9 ± 0.4** **Group 2: 14.8 ± 0.9** **Group 3: 11.2 ± 1.2** **Group 4: 13.0 ± 1.0** **Group 5: 13.3 ± 0.8** **Group 6: 13.0 ± 0.8** **Group 7: 14.3 ± 2.6**	**15.5 ± 9.8**	**18.0 {3.0-34.0}**
Mean ± sd or median {range} glycosylated haemoglobin / Hba1c	**NS**	**No microalbuminuria: 7.9 ± 1.2** **Microalbuminuria: 8.7 ± 1.6**	**Group 1: 11.2 ± 0.2** **Group 2: 13.1 ± 0.3** **Group 3: 12.4 ± 0.7** **Group 4: 12.7 ± 0.4** **Group 5: 12.2 ± 0.4** **Group 6: 12.8 ± 0.7** **Group 7: 12.8 ± 0.8**	**10.1 ± 2.2**	**10.2 {6.7-16.6}**
Number with retinopathy (%)	**NS**	**No microalbuminuria: 8 (47)** **Microalbuminuria: 12 (75)**	**NS**	**7 (22)**	**25 (76)**

	Bognetti *et al.* (1994)	Dash and Torffvit (2003)	Garg *et al.* (1990) [e]	O'Brien *et al.* (1995)	Watts *et al.* (1989)
Exercise test workload	**5 km/hr on treadmill**	**Fixed workload: 150W bicycle at 60 rpm (N= 22) Fixed heart rate: maintained at 80% of max heart rate on bicycle (N = 20) [c]**	**Vigorous pedalling on exercise bike**	**Treadmill with 12% gradient achieving double resting heart rate**	**Treadmill with 12% gradient achieving double resting heart rate**
Exercise test duration (mins)	**15**	**30**	**20**	**20**	**20**
Definition of post-exercise proteinuria	**Albumin excretion rate > 20.1 ug/min**	**NS**	**Albumin excretion rate > 41 ug/min**	**Albumin excretion rate > 30 ug/min [f]**	**Albumin excretion rate > 15 ug/min**
Mean ± sd or median {range} pre-exercise proteinuria of cohort	**4.7 {0.1-15.7} ug/min**	**No microalbuminuria: 1.9 {1.0-60.0} mg/l Microalbuminuria: 7.3 {1.0-115.0} mg/l**	**Group 1: 5.5 ± 0.3 ug/min Group 2: 230.4 ± 56.9 ug/min Group 3: 9.3 ± 1.8 ug/min Group 4: 7.2 ± 0.9 ug/min Group 5: 8.5 ± 1.1 ug/min Group 6: 19.0 ± 5.1 ug/min Group 7: 9.0 ± 2.3 ug/min**	**0.4 {0.1-1.9} mg/mmol**	**4 {0.5-9.4} ug/min**
Mean ± sd or median {range} PeP of cohort	**7.8 {1.3-93.0} ug/min**	**For fixed workload: No microalbuminuria: 50 {11-281} ug/min Microalbuminuria: 102 {17-357} ug/min For fixed HR: No microalbuminuria: 158 {44-2811} ug/min Microalbuminuria: 36 {7.9-496} ug/min**	**Group 1: 8 ± 0.4 ug/min Group 2: 399 ± 111.7 ug/min Group 3: 82.1 ± 13.6 ug/min Group 4: 15 ± 3.2 ug/min Group 5: 63.4 ± 9.3 ug/min Group 6: 52.7 ± 24.5 ug/min Group 7: 72.7 ± 66.1 ug/min**	**NS**	**6.2 {0.6 -96.2} ug/min**
Number of subjects with PeP (%)	**21 (25)**	**NS [d]**	**61 (33)**	**6 (19)**	**10 (30)**
Follow up period mean ± sd or median {range} years	**5.9 ± 1.3**	**13.1 ± 3.2**	**4.0**	**10.0**	**2 {1.5-2.9} [g]**
Definition of follow up primary outcome	**Resting overnight albumin excretion rate between 20-200 ug/min**	**Early morning microalbuminuria persistently > 30 mg/l OR need for haemodialysis**	**Elevated overnight albumin excretion rate of > 30 ug/min on at least two urine samples**	**Early morning resting albumin excretion rate >15 ug/min on three separate days [f]**	**Resting overnight albumin excretion rate > 1 mg/mmol compared to baseline**
Result of follow up primary outcome in PeP positive.	**3/15 (20%) had higher resting albumin excretion rate**	**14/33 (42%) had raised early morning microalbuminuria 2/33 required haemodialysis**	**9/61 (15%) had elevated overnight albumin excretion rate**	**4/6 patients (80%) had a high resting A:Cr**	**4/10 (40%) had > 1mg/mmol in A:Cr**
Result of follow up primary outcome in PeP negative.	**5/46 (11%) had higher resting albumin excretion rate**	**NS**	**0/122 (0%) had elevated overnight albumin excretion rate**	**1/27 (4%) had a high resting A:Cr**	**0/23 (0%) had > 1mg/mmol in A:Cr**

	Bognetti *et al.* (1994)	Dash and Torffvit (2003)	Garg *et al.* (1990) [e]	O'Brien *et al.* (1995)	Watts *et al.* (1989)
Odds ratio for development of CKD if PeP positive [h]	**2.3**	**Unable to calculate**	**42.2**	**52**	**30.7**
Associations with primary outcome	**Nil**	**PeP higher in fixed heart rate compared to fixed workload in those who developed nephropathy at follow up (16/33; 49%)**	**NS**	**PeP was positively correlated with change in A:Cr at 10 years (r = 0.37; *P* = 0.017); PeP was a good predictor of resting A:Cr at 10 years (*P* = 0.005; r2 = 0.31)**	**Nil**
Follow up secondary outcome	**Systolic/diastolic blood pressure and Hba1c**	**Systolic/diastolic blood pressure and Hba1c**	**Hba1c**	**Systolic blood pressure and Hba1c**	**Nil**
Results of secondary outcome	**Hba1c was positively correlated to change in albumin excretion rate (r = 0.293; *P* < 0.05)**	**Maximum systolic blood pressure post-fixed heart rate test was higher in group with microalbuminuria at baseline**	**Nil**	**Baseline Hba1c significant predictor of follow up A:Cr (*P* = 0.02)**	**Nil**

Table 3.3. Data extracted for studies that met criteria for systematic review. [a] Original cohort was 83 but 12 patients lost at follow-up. There were no baseline or PeP differences between lost and follow-up populations, however. Also 5 patients were excluded from the start as pre-exercise proteinuria was >20 ug/min. Therefore, group for final analysis consisted of 66 subjects. [b] Cohort was split into two groups: with or without microalbuminuria at baseline. [c] As part of study aim, fixed workload and fixed heart rate exercise tests were compared. Diabetics were all tested together: 12/22 with no microalbuminuria on fixed workload, 13/20 with no microalbuminuria on fixed heart rate. [d] Unable to determine number who developed PeP as no cut-off was defined. [e] Authors split cohort into seven groups depending on their exercise and overnight albumin excretion rate values. Only group 6 exhibited PeP and elevated resting albumin excretion rate on follow-up. See Garg *et al*. (1990) for further details. [f] Authors considered PeP and rest A:Cr as >4.3 and >2.1 mg/mmol as equivalent to >30 ug/min and 15 ug/min, respectively. [g] Follow-up on focussed on PeP cohort's results. [h] Where there was 0 participants in a category for cacluation of odds ratio, this was replaced by 0.5 (Glas *et al.*, 2003). NS, not specified; PeP, post-exercise proteinuria; A:Cr, albumin-to-creatinine ratio.

	Bognetti *et al.* (1994)	Dash and Torffvit (2003)	Garg *et al.* (1990)	O'Brien *et al.* (1995)	Watts *et al.* (1989)
Methods of patient selection described	Y	Y	Y	Y	Y
Consecutive or random sampling	N	N	N	Y	N
Case-control design avoided	N	N	Y	Y	N
Excluded patients described	N	N	N	N	Y
Avoid inappropriate exclusions	U	Y	Y	Y	Y
Selection of patients bias					
Concern that included patients do not match review question					
Index test described	Y	Y	N	Y	Y
Index test results independently interpreted without reference test	Y	Y	N	Y	Y
Threshold pre-specified	Y	N	Y	Y	Y
Enough information to repeat test in independent study	N	N	N	Y	Y
Index test interpretation bias					
Concern that index test does not match review question					
Reference standard described	Y	Y	Y	Y	Y
Reference standard correct for target condition	Y	Y	Y	N	N
Reference standard results independently interpreted	Y	Y	Y	Y	Y
Enough information to repeat reference test in independent study	Y	Y	Y	Y	Y
Reference standard interpretation bias					
Concern that target condition does not match review question					
Appropriate interval between index and reference tests	N	N	N	Y	N

	Dash and Torffvit (2003)	Garg *et al.* (1990)	O'Brien *et al.* (1995)	Watts *et al.* (1989)	Bognetti *et al.* (1994)
Reference standard for all patients	Y	Y	Y	Y	Y
Same reference standard for all	Y	Y	Y	Y	Y
All patients included in analysis	N	Y	N	Y	Y
Patient flow bias					

Table 3.4. Bias assessment using QUADAS-2 tool for all studies meeting criteria for systematic review. Extra signalling questions were included (see appendices 2 and 3 for elaboration). Green boxes denote low risk of bias/ concerns regarding applicability. Red boxes denote high risk of bias/concerns regarding applicability. N, no; Y, yes; U, unsure.

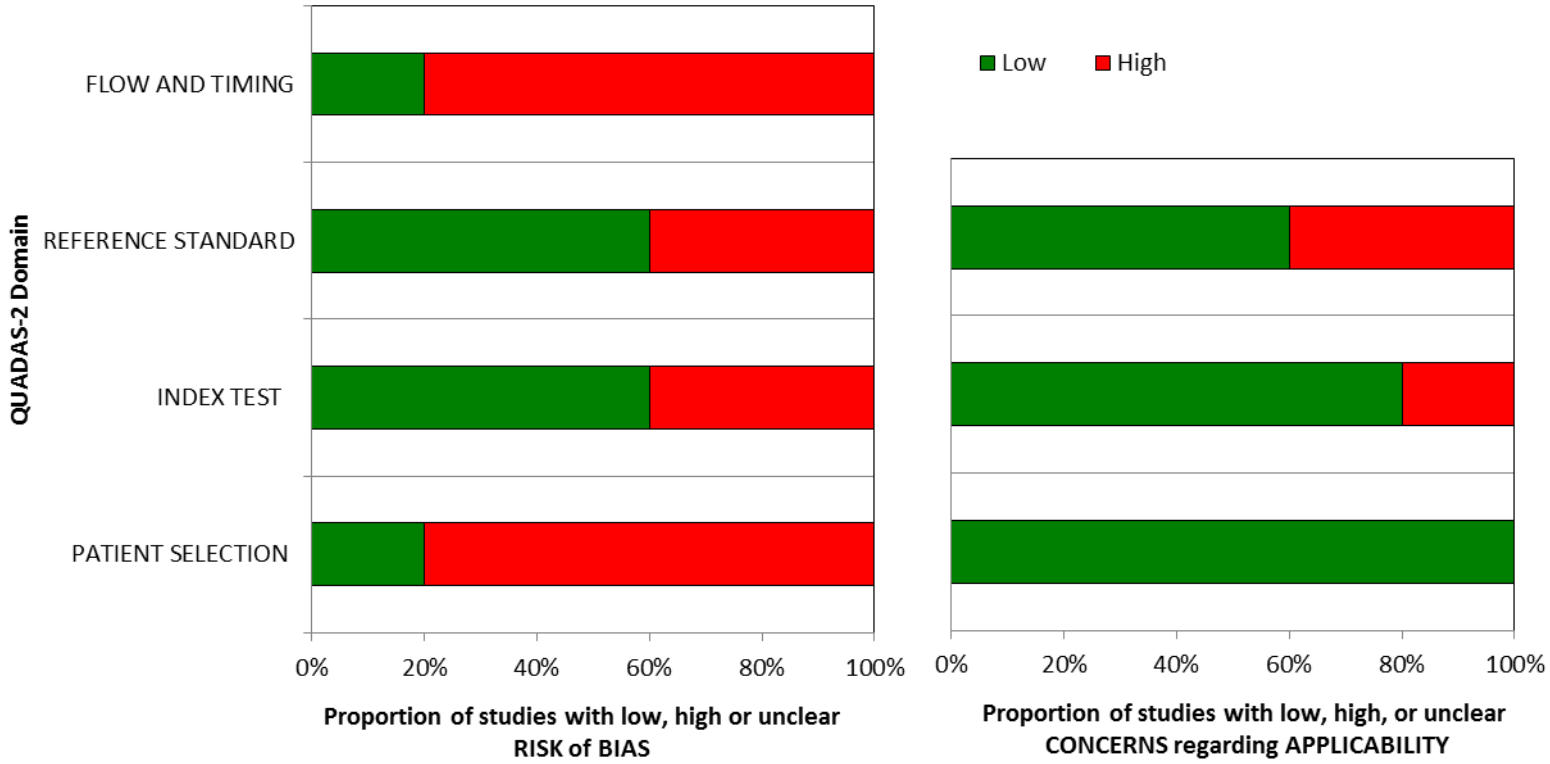

Figure 3.3. Bar graphs summarising risk of bias and concerns of applicability across studies included for systematic review following assessment with QUADAS-2 tool. Green boxes denote low risk of bias/ concerns regarding applicability. Red boxes denote high risk of bias/concerns regarding applicability.

Narrative synthesis. Type of CKD investigated: All studies only examined prospective cohorts with type I diabetes that were at risk of diabetic nephropathy as evidenced by sub-optimal long-term diabetic control (Hba1c ranging from 7.9 to 13.1%). Population demographics: All but one study (Garg *et al.*, N = 187) included less than 100 subjects but no study performed power calculations to determine the sample size required for their primary outcome measure of CKD progression. The age of subjects was in the younger spectrum with the average age ranging from around 15 to 30 years old. Male sex featured predominantly in most studies: only Garg *et al*. demonstrated an approximately equal split between males and females (table 3.3.). Ethnicity was not stated in any study. Although body mass index (BMI) was not specified for three studies, of the remaining two (O'Brien *et al.* and Watts *et al*.) BMI averaged 24 kg/m^2. The duration of diabetes ranged between 8.9 to 18 years. Microvascular complication in the form of retinopathy was accounted for in three studies (Dash and Torfvitt, O'Brien *et al.* and Watts *et al.*) and ranged between 22 to 76% of cohorts.

Exercise test: The exercise intervention used to yield proteinuria varied greatly between all five studies. Except for one study (Dash and Torfvitt), all took into account exercise waterloading to maximise diuresis post-exercise to facilitate proteinuria estimation. This took the form of a single bolus of water (e.g. 500 mls post-exercise) or several boluses evenly spread over a specified time-period prior to commencing exercise. Despite this, no study measured the effectiveness of waterloading upon hydration status (e.g. through urine osmolality, specific gravity or urine colour). For their exercise protocol, two studies (Dash and Torfvitt and Garg *et al.*) used exercise ergometers and the remaining three used treadmills. There was little information on how exercise was tailored to the patient with respect to intensity: Bognetti *et al*. stated that their protocol was of "moderate exercise" whilst Garg *et al.* used 20 minutes "vigorous pedalling on an exercise bike", with neither author providing quantification on how these exercise intensities were determined. The remaining three studies used increments of resting heart rate as a measure of intensity and Dash and Torfvitt compared intensity prescriptions by comparing fixed workload and fixed heart rate regimes. The duration of exercise varied between 15 to 30 minutes.

With regards to post-exercise measurements, two studies (Dash and Torfvitt, Garg *et al.*) took proteinuria measurements immediately following the exercise bout. Bognetti *et al.* chose the highest value of several measurements post-exercise while O'Brien *et al.* took a sample 20 minutes post-exercise. Watts *et al.* did not specify when their measurement(s) was / were taken. The cut-off for significant PeP was generally defined as above the limit for significant (pathological) albuminuria at rest as set by contemporary consensus guidelines in diabetic

nephropathy. This predated KDIGO guidance in all cases, but Dash and Torfvitt did not state any limit for significant PeP. The proportion of inidividuals with PeP from the cohort varied between 19 to 33%.

Follow-up: The follow-up period for all studies was beyond three months and ranged between 2 to 13.1 years. Primary outcome: For all studies, primary outcomes concentrated on CKD progression as defined by higher-than-resting values of albuminuria at follow-up. Only one study (Dash and Torfvitt), measured serum Cr pre exercise and at follow-up and noted that the median concentration was lower at follow-up (80 {65-102} umol/l vs. 75 {53-317} µmol/l, respectively). Estimated GFRs were not calculated however. The proportion of individuals with PeP with significant albuminuria at follow-up varied between 5 to 80%, though no study clarified if follow-up measurements were taken in a steady-state period with other medical conditions potentially contributing to proteinuria being excluded. Moreover, during the follow-up period in one study (Dash and Torfvitt), it was noted that 10 patients had commenced proteinuria-modifying medications (e.g. angiotensin-converting enzyme inhibitors). There was no information to support a similar finding in the remaining studies. Only O'Brien *et al.* concluded that over a 10-year follow-up period, PeP was a good predictor of resting A:Cr at follow-up and used regression analysis to take into account confounding factors affecting proteinuria (r^2 = 0.31; P = 0.005). The remaining four studies did not individually demonstrate any statistically significant associations between PeP and proteinuria at follow-up. However, when combining the results of the primary outcome in four studies (Bognetti *et al*., Garg *et al.*, O'Brien *et al.* and Watts *et al*.), the presence of PeP was highly associated with elevated resting proteinuria at follow-up (χ^2 test, $P < 0.0001$). Moreover, significant odds ratios (developing CKD following a positive exercise test vs. not developing CKD) was found for each of the four studies, ranging between 2.3-52.0. Secondary outcomes: Four studies (Bognetti *et al*., Dash and Torfvitt, Garg *et al.* and O'Brien *et al.*) also investigated secondary outcomes that included other modifiable CKD risk factors related to diabetic nephropathy such as Hba1c and hypertension: with respect to the primary outcome measure, Bognetti *et al*. noted that Hba1c was positively correlated to change in albumin excretion rate (PeP to follow-up resting proteinuria).

3.5. *DISCUSSION*

Summary of evidence. This is the first systematic review to investigate whether exercise-induced proteinuria can predict the onset or progression of CKD in humans, and demonstrated a paucity of studies in this area as assessed over a 60-year period. Of the small number of studies that do exist, the focus was on young (predominantly male) cohorts with insulin-dependent (type 1) diabetes at-risk of incipient nephropathy. Comparison by meta-analysis was deemed not feasible given the great variability in the interventions used to generate PeP and timing of measurements. Despite these issues, results for the primary outcome of progression to CKD from four studies were pooled together. From this, chi-squared analysis and odds ratios for the development of CKD given presence of PeP were both found to be highly significant, suggesting that exercise testing could be a useful predictive tool for CKD progression. Additionally, of the five studies included for review, one study demonstrated a positive association between the presence of PeP and development of CKD (as defined by elevated resting albuminuria) over a 10-year period.

Limitations. Study limitations: Given the viewpoint that diabetic nephropathy is the leading cause of end-stage renal disease in the Western World, it is not unexpected that all eligible studies focussed on diabetic patients at-risk of secondary nephropathy (Harjutsalo and Groop, 2014). Other plausible explanations for an emphasis on diabetics includes ease of sampling and familiarity as previous work examining PeP in diseased states has largely focussed on populations with diabetes (Poortmans 1984). Thus, all studies were rated as having low concern regarding applicability of the patient selection to the review question. Nonetheless, to determine if exercise-testing will be a useful predictor of CKD in general, future studies must focus on populations regardless of their primary disease or their cause of CKD. From a biological perspective this is plausible as the biochemical and cellular aberrations that drive CKD towards advancing glomerulosclerosis and the treatments used to prevent its progression (e.g. angiotensin-receptor blocking agents) are similar regardless of the underlying aetiology of CKD (Oite 2011). Using general CKD populations would also serve to increase sample size and are more likely to confidently achieve power calculations for primary outcome measurements.

The studies examined also had a heavy bias towards a younger population (average age ranging from 15 to 30 years). However, it is well known that the prevalence of CKD is greater in people over 60 years: between the 1988 to 1994 National Health and Nutrition Examination Survey (NHANES) study and the 2003 to 2006 NHANES study, the prevalence

of CKD in people 60 years and above rose from 18.8 to 24.5%, whereas during the same period, the prevalence of CKD in people between the ages of 20 and 39 remained consistently below 0.5% (de Boer *et al.*, 2011). Moreover, renal responses to exercise are blunted with advancing age (Walker *et al.*, 1994). Thus, he older age groups are set to benefit the most from such a test and the results (and thus exercise protocol) of the current studies are of questionable applicability to this population. Future study designs should take these factors into account.

As reflected by the QUADAS-2 scores, the interventions (index test) used across studies harbour a significant source of study bias with some concerns of applicability (figure 3.3.). The intensity and duration of exercise are two key determinants in yielding PeP (Poortmans 1984). For example, Poortmans *et al.* (1982) remarked that an 800 metre sprint had the highest propensity for exercise proteinuria and this can reliably yield exercise proteinuria in healthy individuals (Junglee *et al.* 2012). Thus, high-intensity and short duration routines are likely to maximise post-exercise proteinuria. The studies examined used exercise routines, which although convienent for a clinical setting, varied in intensity and duration. Addtionally, hydration to ensure diuresis and the timing for measurement of PeP was also variable despite previous work showing that hydration status could affect baseline measurements and that maximal proteinuria occurs 20-30 minutes post-exercise (Poortmans 1984; Junglee *et al.* 2012). These factors introduce interpretation bias and make fair comparison between studies (and hence meta-analysis) difficult. Future studies require replicable and comparable exercise interventions that are tailored to patients using a well-established and robust measure of intensity (e.g. $\dot{V}O_{2max}$). This and other important factors are summarised in table 3.5. Diseased individuals may show exaggerated responses to exercise and if all participants experienced PeP, they could potentially be further catagorised depending on their level of PeP (i.e. microalbuminuric versus macroalbuminuric).

Factors to consider when developing an exercise proteinuria provocation test
Intensity of exercise determined with valid subject specific measure (e.g. % $\dot{V}O_{2max}$).
Short duration activity e.g 5 to 10 minutes.
Adequate pre-hydration and checked using urine osmolality or colour.
Robust methods to ensure significant resting proteinuria is excluded prior to testing e.g. 3 consecutive early-morning urine samples negative for proteinuria.
Cut-off for significant proteinuria is based upon pathological criteria.
Methodology is replicable and inexpensive.

Table 3.5. Some of the factors that should be considered when developing an exercise test for proteinuria provocation.

Outcome limitations: All studies chose follow-up periods upto an average of 13 years, though within a recognised time-frame where diabetic nephropathy type I diabetics may develop (15-25 years from diagnosis; Mauer *et al.*, 1997). Nevertheless, such lengthy follow-up testing for CKD could be subject to confounding by a number of factors such as introduction of proteinuria-modifying treatments (e.g. angiotensin-receptor antagonists) and the development of other nephropathies (e.g. IgA nephropathy). This is reflected by the large proportion of high bias risk across studies for the flow and timing domain of QUADAS-2 (figure 3.3.).

In most studies, there was consideration given for changes in diabetic control with Hba1c measurements at baseline and follow-up, but only O'Brien *et al.* made an attempt to correct for a range of confounders by regression analysis and they demonstrated a positive association of post-exercise proteinuria and elevated resting proteinuria after a 10-year follow-up. Although it is appreciated in the real-life setting such factors are difficult to control for, it is recommended that future study should consider appropriate statistical correction to account for these analysis (e.g. multiple regression).

With respect to the primary outcome measure, definitions of pathological proteinuria at rest were generally acceptable with contemporary consensus opinions – largely in keeping with later KDIGO guidance (Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group, 2013). However, it was surprising to find only one study documenting use of serum Cr as a primary outcome measure for CKD progression. Serum Cr is the most commonly used biomarker of CKD and would have been accessible in the time period when all five studies were conducted (1989 to 2003; Levey *et al.*, 1999). Although arguably more invasive than a urine sample, it would be subject to less variability / labour-cost compared to A:Cr ratio estimations (which have to be repeated on three morning occasions) and can be incorporated into estimated GFR calculations. Additionally, all studies did not clarify explicitly that follow-up measurements were taken in a steady state period as the presence of intercurrent illness and previous exercise can influence proteinuria.

Review limitations: A drawback of the review process includes the lack of grey-area searching and exclusion of non-English language literature. However, key electronic databases were searched, a broad scope in terminology was permitted to maximise hits and a medical librarian was utilised. Despite attempts, it was not possible to retrieve missing information from study authors (e.g. from Dash and Torfvitt), but regardless it is likely this would not significantly change the key findings of this review. Meta-analysis was not possible given the high degrees of bias in most studies and the variability in the interventions. The results from this systematic review are only applicable to type I diabetics at risk of CKD progression and cannot be used to make generalisations to the CKD population as a whole.

3.6. *CONCLUSION*

Despite the limited number of studies in the literature and their shortcomings, post-exercise proteinuria shows promise; as currently evidenced in a type I diabetic population. This is supported by highly significant odds ratios for four studies included in the systematic review. However, there is a need to refine exercise interventions and such findings should provide enough impetus for this before applying them to wider at-risk CKD populations.

3.7. *FUNDING*

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CHAPTER 4

EXERCISING IN A HOT ENVIRONMENT WITH MUSCLE DAMAGE: EFFECT ON ACUTE INJURY BIOMARKERS AND KIDNEY FUNCTION

4.1. *ABSTRACT*

Unaccustomed strenuous physical exertion in hot environments can result in exertional heat stroke and acute kidney injury (AKI). Both exercise-induced muscle damage and AKI are associated with the release of interleukin-6 (IL-6), but whether muscle damage causes AKI in the heat is unknown. It was hypothesized that muscle damaging exercise, prior to exercise in the heat would increase kidney stress. To test this hypothesis, ten healthy, euhydrated males underwent a randomized, crossover trial involving both a 60 minute downhill muscle-damaging run (EIMD), and an exercise intensity-matched non-muscle damaging flat run (CON), in random order, separated by two weeks. Both treatments were followed by heat stress elicited by a 40 minute run at 33^{o}C. Urine and blood were sampled at baseline, after treatment (EIMD or CON), and immediately after running in the heat. As expected, EIMD induced higher plasma creatine kinase and IL-6 than CON ($P < 0.001$ and $P = 0.005$, respectively). EIMD elevated kidney injury biomarkers (e.g. urinary NGAL after running in the heat: EIMD–CON, mean difference [95% CI]: 12 [5, 19] ng/ml) and reduced kidney function (e.g. plasma creatinine after running in the heat: EIMD–CON, mean difference [95% CI]: 0.2 [0.1, 0.3] mg/dl). Plasma IL-6 was positively correlated with plasma NGAL ($r = 0.9$, $P = 0.001$). Moreover, following EIMD, 5 of 10 participants met Acute Kidney Injury Network criteria for AKI. Thus, for the first time it is shown that muscle damaging exercise prior to running in the heat results in greater inflammation and kidney stress compared to non-muscle damaging exercise. Muscle damage should therefore be considered a risk factor for AKI when performing exercise in hot environments.

4.2. *INTRODUCTION*

Unaccustomed strenuous exercise in the heat may lead to exertional heat injury (Adams *et al.*, 2012). This has been reported in athletes, military personnel, and those in other hazardous occupations such as firefighters (Carter *et al.*, 2005; Howe and Boden, 2007; Wallace *et al.*, 2007). Exertional heat illness exists as a spectrum, which ranges from relatively mild exertional heat injury to life-threatening heat stroke with reduced consciousness and multi-organ failure (Carter *et al.*, 2005). In 2011, there were approximately 3000 cases of exertional heat injury in US military soldiers with over 10% sustaining heat stroke (MSMR, 2012).

An association between heat stroke and kidney dysfunction has long been recognized, and although not universal, this combination appears to exacerbate the risk of severe acute kidney injury (AKI). This itself portends significant mortality and may require supportive treatment in the form of renal replacement therapy e.g. haemofiltration (Schrier *et al.*, 1967; Raju *et al.*, 1973; Lin *et al.*, 2003). Treatment for severe AKI can be protracted and full recovery is not guaranteed: survivors of this catastrophic condition may be left with chronic kidney disease which itself represents a significant morbidity (Okusa *et al.*, 2009).

Observations, mainly from longitudinal studies and case reports, suggest that there are a number of factors that are potentially associated with the development of heat illness including a hot environment, dehydration, use of non-steroidal anti-inflammatory agents, and poor fitness levels (Lin *et al.*, 2003; Carter *et al.*, 2005; Howe and Boden, 2007; Leon and Helwig, 2010; Adams *et al.*, 2012; Fortes *et al.*, 2013). Some theories also propose that immune dysregulation is partly responsible for the development of heat stroke. In this context, immune dysregulation involves an elevation of pro-inflammatory cytokines including the pleiotropic cytokine interleukin-6 (IL-6) and vasoconstricting mediators (Fortes *et al.*, 2013).

There is also evidence that AKI *per se* represents a pro-inflammatory condition with cytokines released by leukocytes and renal tubular cells in the injured kidney. These molecules are believed to be important components of both the initiation and extension of kidney injury (Lee *et al.*, 2012). Recent findings suggest that IL-6 plays a pivotal role in the development of AKI, by promoting its early development via an injurious inflammatory reaction (Nechemia-Arbely *et al.*, 2008; Lee *et al.*, 2012). This may be related to trans-signaling and "Signal Transducers and Activators of Transcription" protein activation in renal tubular cells (Lee *et al.*, 2012).

Interestingly, strenuous unaccustomed exercise can result in muscle damage and consequently elevated circulating IL-6. (Philippou *et al.*, 2009; Montain *et al.*, 2000; Fortes *et*

al., 2013; Pedersen 2013). Thus, it is plausible that completing exercise in hot environments with muscle damage could generate a more severe pro-inflammatory response that induces kidney dysfunction typical of severe heat illness. The recent discovery of novel biomarkers of AKI offers more sensitive and specific method of investigating the kidney in experimental models of heat stress. One such biomarker, that has been used in a variety of AKI settings, is neutrophil gelatinase-associated lipocalin (NGAL) (Mishra *et al.*, 2003; Mishra *et al.*, 2004; Mishra *et al.*, 2005; Mishra *et al.*, 2006; Nickolas *et al.*, 2008). Combined with traditional markers of kidney function, such as creatinine (Cr) filtration and urine output, a more complete understanding of the kidney's stress response can be gained. Therefore, the unknown contribution of the pro-inflammatory state resulting from muscle damage on kidney function during heat exposure can, for the first time, be investigated.

Consequently the aim of this study was to investigate the effect of muscle damage and subsequent exercise in the heat on markers of kidney injury and function. It was hypothesized that following a bout of muscle damaging exercise, during subsequent exercise in a hot environment: 1) kidney injury biomarkers would be upregulated (e.g. increased urine and plasma NGAL concentrations); 2) kidney function would be reduced (e.g. plasma Cr concentration will be increased); and 3) that changes in circulating interleukin-6 and consequently biomarkers of kidney injury would be correlated with changes in kidney function.

Participants. The study was undertaken in accordance with the Declaration of Helsinki (2008) and the institutional ethics committee gave ethical approval. Recruitment occurred between January 2011 and August 2011. Potential participants who were aged between 18 to 30 years and recreationally active were approached via University and local sports clubs. Ten healthy Caucasian male subjects were successfully enrolled (mean age ± SD, 20 ± 2 years). All provided written consent and none had medical conditions, musculoskeletal injury, history of heat illness, or medication use (e.g. regular use of non-steroidal anti-inflammatory drugs) that precluded participation. Moreover, none of the subjects were heat-acclimatized or regularly participated in sports/activities that involved large eccentric movements of the legs (e.g. mountain running, weight lifting). A day prior to each trial, subjects avoided heavy unaccustomed exercise and consumption of alcohol or caffeine.

Study Design. A counterbalanced crossover design was employed, with each participant performing exercise in the heat following a treatment that consisted of either muscle damage exercise (EIMD; running downhill on a -10% gradient treadmill) or exercise intensity-matched but non-muscle damaging exercise (CON; running on +1% gradient treadmill; figure 4.1). All subjects performed both treatments, in random-order, on separate occasions with a 14-day washout period between treatments. Order of presentation of treatments was randomized by using on-line software (http://www.randomizer.org; Site Statistics, Social Psychology Network) with blinding to the identities and demographic details of all participants.

Familiarization and Maximal Exercise Testing. At least one week prior to the first experimental trial, maximal oxygen uptake ($\dot{V}O_{2max}$) was assessed by a continuous incremental exercise test on a motor driven treadmill (Mercury 4.0, HP Cosmos, Nussdorf-Traunstein, Germany). Participants ran initially at 8 km/h at a constant 1% gradient with the running speed increased by 2 km/h every three minutes. At 16 km/h the gradient was increased by 2.5% every three minutes until volitional exhaustion. During this entire period, oxygen uptake and carbon dioxide production were determined using an on-line breath-by-breath system (Cortex Metalyser 3B, Biophysik, Leipzig, Germany). Using linear interpolation of the running speed versus $\dot{V}O_2$ relationship, the speed eliciting 65% $\dot{V}O_{2max}$ was calculated. Following a rest period of 15 minutes, a four minute speed verification test was conducted to identify the speed that would evoke 65% $\dot{V}O_{2max}$ whilst running at +1% and

-10% gradients. Running speed was adjusted to elicit 65% $\dot{V}O_{2max}$ and verified by concomitant measures of expired gas; thus ensuring exercise intensity was the same on both gradients in the experimental arms.

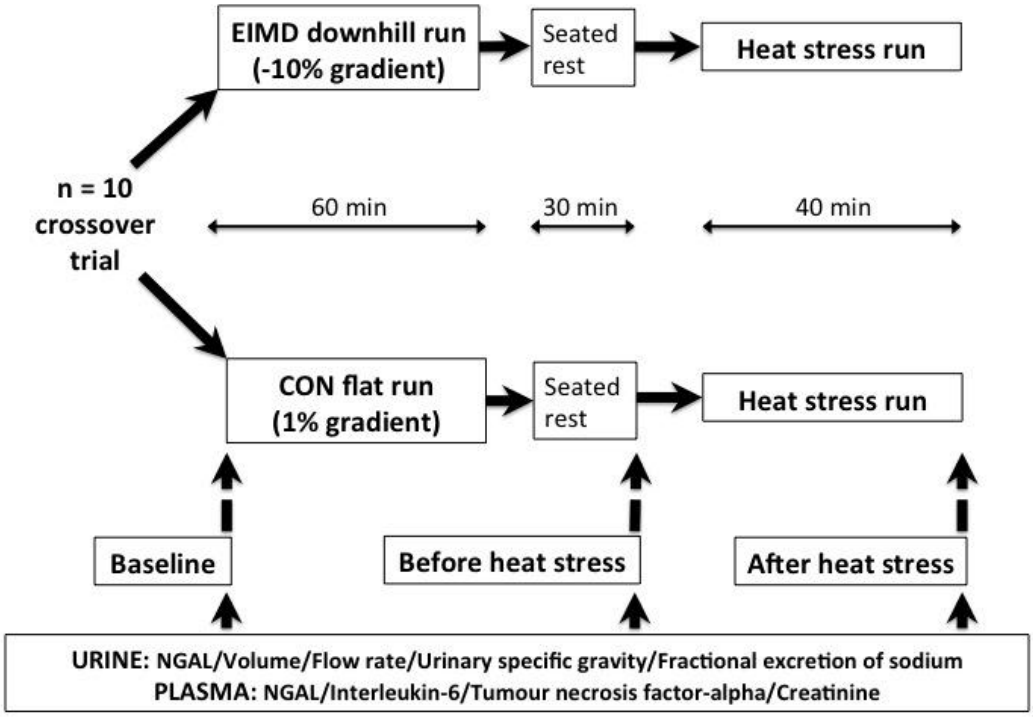

Figure 4.1. Schematic detailing flow of study. Treatment (EIMD or CON) was performed at 20 ^{o}C. Heat stress was performed at 33 ^{o}C. Baseline, before heat stress and after heat stress denote sampling time points. EIMD, exercise-induced muscle damage arm; CON, control arm; NGAL, neutrophil gelatinase-associated lipocalin.

Dietary Control. All participants had a standardized breakfast before the start of each trial (cereal bar; 2 kcal/kg body mass). Furthermore, subjects were requested to replicate the diet consumed 24 hours before the start of each treatment arm; this was confirmed through written diet diaries. Euhydration was achieved by subjects drinking 40 ml/kg nude body mass (Model 705, Seca, Hamburg, Germany) of water during the 24 hours before a treatment arm, and consuming an additional 5 ml/kg nude body mass approximately 30 minutes before. To confirm euhydration, urine specific gravity was measured using a handheld refractometer (Atago Uricon-Ne refractometer, NSG Precision cells, New York, USA) on the initial urine sample on the day of study (Armstrong *et al.*, 1998).

Experimental Protocol (Treatment). For both EIMD and CON conditions, participants wore standardized clothing consisting of running shorts, socks and shoes and performed a

warm-up consisting of a three-minute walk on the treadmill at 5 km/h. Depending on the treatment allocation, subjects then ran at their verified speed to elicit 65% $\dot{V}O_{2max}$ on either a -10% gradient treadmill (EIMD) or a +1% gradient treadmill (CON) in an ambient temperature of 20 °C and 40% relative humidity for 60 minutes. During this period, participants consumed water (2.5 ml/kg nude body mass) at 0, 15, 30 and 45 minutes. Following this exercise bout, subjects sat in ambient conditions for 30 minutes and a single bolus of water equivalent to 5 ml/kg nude body mass was administered.

Heat stress. Exercise in the heat was then completed, which involved running on the treadmill at a 1% gradient for 40 minutes at 65% $\dot{V}O_{2max}$ in an environmental chamber (Delta Environmental Systems, Chester, UK; figure 4.1.) maintained at a dry bulb temperature of 33^{o}C with 50% relative humidity. No fluids were provided during this bout. To conclude the trial, participants were provided with 30 ml/kg body mass fluids to consume in the following 21 hours.

Kidney injury and function. To assess kidney injury, NGAL concentration in blood and urine was determined. To assess kidney function, plasma Cr concentration (filtration), urine volume, urine specific gravity (urine concentration), and fractional excretion of sodium (integrity of renal tubular reabsorptive function) were measured. Sampling time points are shown in figure 4.1. For micturition, participants were asked to maximize bladder emptying through double voiding. Urine volumes were collected at each time point using 24-hour urine containers to determine urine flow rate and to measure urinary specific gravity (see above).

The fractional excretion of sodium was calculated as follows:

$$[(\text{sodium}_{\text{urinary}} \times \text{creatinine}_{\text{plasma}}) \div (\text{sodium}_{\text{plasma}} \text{ x } \text{creatinine}_{\text{urinary}})] \times 100.$$

Whole blood (lithium heparin, K_2EDTA and serum) was taken from the antecubital vein using a 22-gauge needle and Vacutainer (BD, Oxford, UK). Serum tubes were left to stand for one hour before centrifugation. Blood samples were centrifuged for 10 minutes at 1500 *g*. Serum, plasma and urine aliquots were stored at -80 ^{o}C for subsequent biochemical analysis.

Muscle damage and inflammation. As an indicator of muscle damage, plasma creatine kinase was measured at baseline and 24 hours post-treatment – a period where one could reasonably expect and elevation of creatine kinase from any muscle damage sustained during the protocol. Similar to the measurements of kidney function and injury, circulating plasma

IL-6 and tumor necrosis factor alpha (TNF-α) were measured at baseline and pre- and post-heat stress as markers of inflammation (figure 4.1.).

Biochemical Analyses. Enzyme-linked immunosorbent assays (ELISA) were performed for the following analytes using commercially-based kits as per kit instructions: plasma creatine kinase (EnzyChrom creatine kinase assay kit, BioAssay Systems, Hayward, USA); plasma IL-6 (Quantikine high sensitivity IL-6, HS600B, R&D Systems Europe, Abingdon, UK); plasma TNF-α (High Sensitivity TNF-α ELISA with Signal Amplification, eBioscience, Vienna, Austria) and plasma / urinary NGAL (NGAL Rapid ELISA kit, Bioporto, Gentofte, Denmark). Absorbances were read at a wavelength of 450 nm by a microplate reader (FLUOstar Omega, BMG Labtech GmbH, Ortenburg, Germany). Intra-assay coefficient of variation (CV) for creatine kinase, IL-6, TNF-α and NGAL (plasma/urine) were 3.8%, 5.3%, 9.5% and 4.7/8.4%, respectively.

Plasma and urinary Cr was measured by the Jaffe method on an Olympus AU2700 automated analyzer (assay: OSR6178; Olympus, UK; Beckmann-Coulter, UK). Plasma and urine sodium was measured by an indirect ion selective electron probe on the same analyzer. For each biochemical analysis, all participant samples were assayed on the same plate.

Urinary NGAL concentrations are presented as absolute values and corrected for urinary flow rates (NGAL ng/min). This correction is an acceptable method to account for changes in urinary concentration and/or dilution. Although normalization of urinary NGAL to urinary Cr is commonly used in clinical and research settings to account for urinary concentration changes (cf. albumin:Cr ratio), its application in exercise models could be flawed. Marked reductions in glomerular filtration rate and tubular dysfunction occur during high-intensity exercise, and subsequently urinary creatinine excretion becomes a non-constant (Poortmans 1984; Junglee et al., 2012). Thus, normalization against urinary flow rate was used instead (Waikar *et al.*, 2010). All blood analytes were corrected for plasma volume shifts (Dill and Costill, 1974).

Exercise intensity. To confirm energy expenditure was the same in both treatment arms, metabolic energy expenditure (M; W/m^2) was determined by the following: oxygen consumption (VO_2) during treadmill running was calculated from 60 seconds expired air samples collected into a Douglas bag which were analyzed for oxygen and carbon dioxide concentrations (SERVOPRO 1440 gas analyzer, Servomex, Crowborough, UK) and volume (Harvard Apparatus, Edenbridge, UK).

This, together with the respiratory exchange ratio (RER; VO_2/VCO_2), and body surface area (BSA) by the DuBois and DuBois (1916) method was used in the following equation:

$$\text{Metabolic energy expenditure (Watts)} = (VO_2 \times (21166 \times [0.23(\text{RER}) + 0.77]) / 60) / \text{BSA}$$

Body temperature and hydration status. Core temperature was measured at each time point by a rectal thermistor inserted 12 cm beyond the anal sphincter (Henleys Medical Supplies Ltd, Herts, UK) and connected securely to a portable data logger (YSI model 4000A, YSI, Dayton, USA).

Hydration status was assessed through nude body mass change and plasma volume change. The latter was determined by measurement of haemoglobin (β-Haemoglobin Hemocue AB photometer, Hemocue Ltd, Dronfield, UK) and haematocrit (Hawksley and sons Ltd, Sussex, UK) measured on whole blood in triplicate and then averaged for subsequent plasma volume shift calculations as described above.

Statistical Analysis. Data for all dependent variables were examined for normality using histogram plots and Shapiro-Wilk tests. All data were found to be normally distributed except for urinary NGAL and flow rate corrections. Thus, mean ± SD are stated for all measures except for urinary NGAL where median {interquartile range} are presented. For the purposes of further statistical analysis, urinary data were successfully transformed to normality using a $\log_{10}$ function.

For all dependent variables, EIMD and CON groups at pre-heat stress and post-heat stress were compared using parametric two-way repeated measures analysis of variance (group vs. time) with adjustments made to the degrees of freedom when assumptions of sphericity were violated. *Post-hoc* Bonferroni adjusted *t*-tests or Tukey's tests were used as appropriate to follow up significant interactions.

Mean differences and 95% confidence intervals between EIMD and CON groups at pre-heat stress and post-heat stress were calculated for all measures. In the case of urinary NGAL and urinary NGAL flow rates, the Hodges and Lehman method was used to derive median differences and 95% confidence intervals between EIMD and CON.

Plasma creatine kinase concentrations in EIMD and CON arms were compared at baseline and 24 hour post-treatment by two-tailed paired Student's t-tests with Bonferroni correction. Mean energy expenditure in the EIMD and CON arms were compared during the respective treatment bout and during exercise in the heat by two-tailed paired Student's t-tests with Bonferroni correction.

To determine the possible relationships between plasma IL-6, measures of renal function and biomarkers of kidney injury, bivariate Pearson's correlations were performed on percentage change scores from baseline to post-heat stress. For all tests, statistical significance was accepted when $P \leq 0.05$. SPSS version 18.0 (SPSS Inc., IBM, Chicago IL, USA) was used for all statistical analysis.

For the primary outcome measure of NGAL, a power calculation revealed that six participants were needed per group to detect a smallest important change in means of 4.6 ng/ml. This value is the increase required to elevate urinary NGAL from the normal healthy population mean (5.3 ng/ml) to above the normal range (0.7-9.8 ng/ml) (NGAL, Bioporto Diagnostics). This power calculation assumes a within subject error of 1.7 ng/ml, and allows for a 5 and 20% chance of making a Type I or Type II error, respectively (Hopkins 2006).

4.4. *RESULTS*

All ten male participants (age 20 ± 2 years; height 176 ± 6 cms; $\dot{V}O_{2max}$ 59 ± 4 ml/kg/min) successfully completed both trials and there were no participant dropouts or complications due to heat illness.

Urinary NGAL and Corrections for Urinary Flow Rate. The heat stress exercise bout elicited a rise in median urinary NGAL concentration following EIMD but not CON exercise (figure 4.2.). At post-heat stress, 8 of 10 individuals and 3 of 10 individuals, in EIMD and CON trials, respectively, demonstrated rises in urinary NGAL concentrations above the normal range,.

Absolute urinary NGAL concentrations were also corrected for urinary flow rates, thus taking into account any alterations in urine production that may confound interpretation of raw values. Between pre- and post-heat stress, CON demonstrated a fall in urinary NGAL flow rate whereas EIMD exhibited a slight rise (baseline, pre-heat stress, post-heat stress for EIMD: 4.6 {2.5-5.6}, 10.3 {4.4, 11.7}, 12.4 {8.7, 19.2} ng/min; for CON: 9.4 {7.8-12.4}, 19.7 {11.6, 29.0}, 8.5 {5.7, 11.7} ng/min). For these data there was a significant interaction (P = 0.002), but no main effects of time or group (P = 0.2 and 0.3, respectively). Mean difference (EIMD – CON) and 95% confidence intervals for urinary NGAL flow rate between both groups were -0.3 [-0.6, -0.1] ng/min and 0.1 [0.007, 0.3] ng/min at pre-heat stress and post-heat stress, respectively.

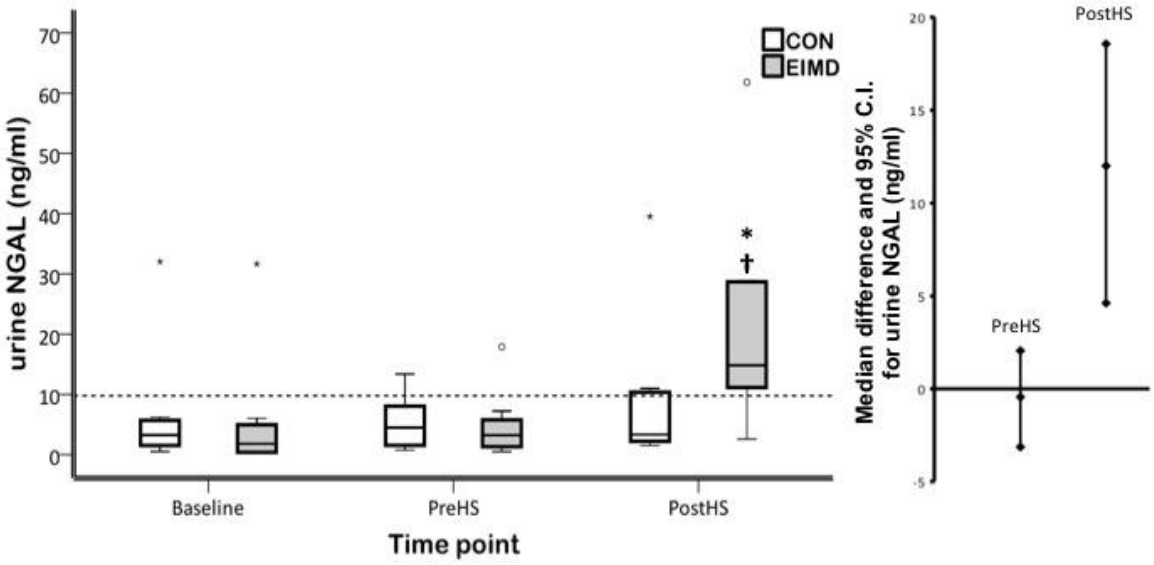

Figure 4.2. Left panel: urinary neutrophil gelatinase-associated lipocalin (NGAL) at baseline, before (preHS) and after (postHS) heat stress for control (CON; clear boxes) and exercise induced muscle damage (EIMD; shaded boxes) groups. Data are presented as median (solid line in box), quartiles (boxes) and ranges (whiskers) given non-parametric data. Circles and small stars indicate outliers. Dotted horizontal line on y-axis indicates upper limit of normal range for urinary NGAL (9.8 ng/ml). For EIMD and CON groups, there was a significant interaction between pre- and postHS (P = 0.005). By post hoc test: *, significantly different from PreHS; †, significantly different from CON. Right panel: median difference and 95% confidence intervals between EIMD and CON for urinary NGAL at pre- and postHS. A positive value indicates urinary NGAL was higher in the EIMD group.

Plasma NGAL. Between pre- and post-heat stress, there were increases in plasma NGAL concentrations that were more prominent in EIMD (figure 4.3).

Plasma creatinine. Plasma Cr increased between pre-heat stress and post -heat stress in both groups, but more so for EIMD (figure 4.4). By post- heat stress, 5 out of 10 participants in EIMD met stage 1 of the Acute Kidney Injury Network (AKIN) criteria (plasma Cr rise of > 0.3 mg/dl from baseline), compared to 0 out of 10 participants in CON (Mehta *et al.*, 2007).

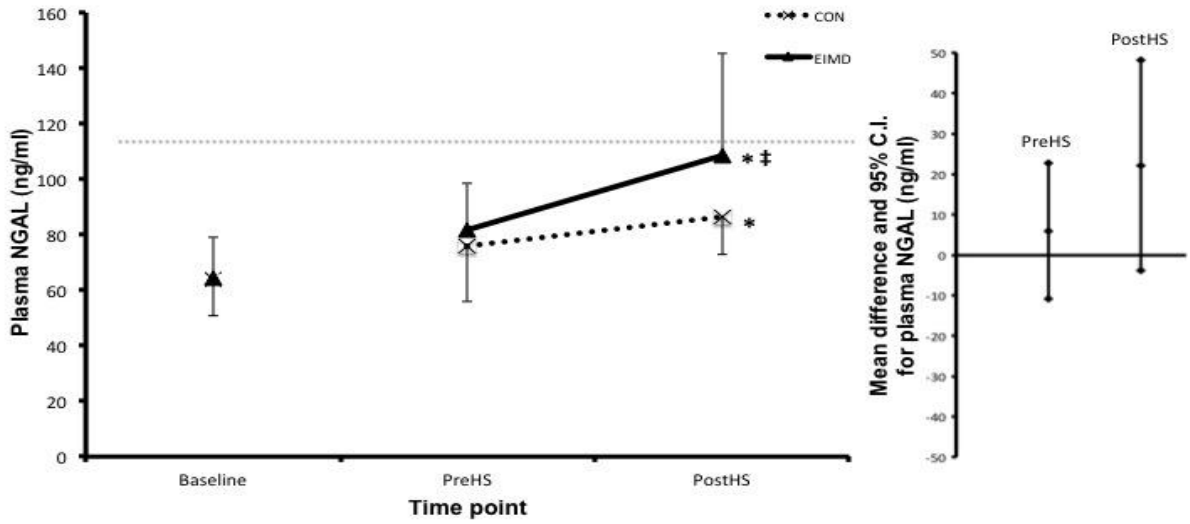

Figure 4.3. Left panel: plasma neutrophil gelatinase-associated lipocalin (NGAL) at baseline, before (preHS) and after (postHS) heat stress for control (CON; dotted line) and muscle damage (EIMD; solid line) groups. Data are means (SD). Dotted horizontal grey line crossing y-axis indicates upper limit of plasma NGAL (106 ng/ml). For EIMD and CON groups, there was a significant interaction between pre- and postHS (P = 0.04). By post hoc test: *, significantly different from PreHS; ‡ trend ($P \geq 0.05$ but ≤ 0.1) for difference with CON. Right panel: mean difference and 95% confidence intervals between EIMD and CON for plasma NGAL at pre- and postHS. A positive value indicates plasma NGAL concentration was higher in the EIMD group.

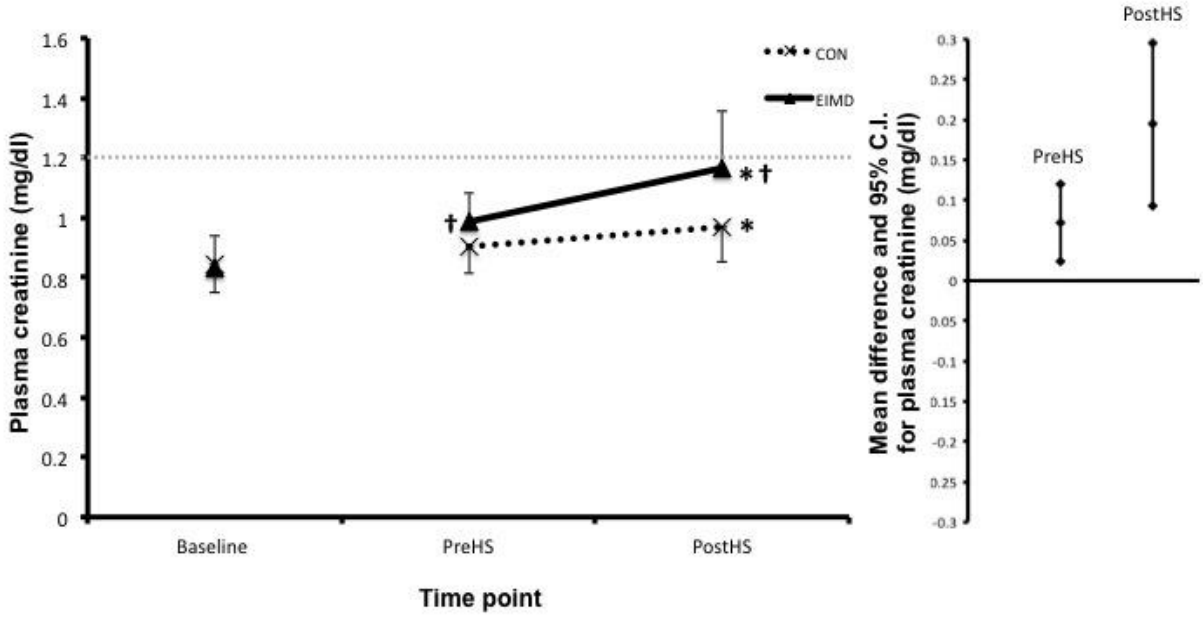

Figure 4.4. Left panel: plasma creatinine at baseline, before (preHS) and after (postHS) heat stress for control (CON; dotted line) and muscle damage (EIMD; solid line) groups. Data are means (SD). Dotted horizontal grey line crossing y-axis indicates upper limit of plasma creatinine (1.2 mg/dl). For EIMD and CON groups, there was a significant interaction between pre- and postHS (P = 0.005). By post hoc test: *, significantly different from PreHS; †, significantly different from CON. Right panel: mean difference and 95% confidence intervals between EIMD and CON for plasma creatinine at pre- and postHS. A positive value indicates plasma creatinine was higher in the EIMD group.

Urinary Volume, Flow Rate and Specific Gravity. Between pre- and post-heat stress, there was a reduction in urine volume that was more pronounced in EIMD, exhibiting significant main effect of time and group ($P < 0.05$ and $P = 0.05$, respectively), albeit no interaction ($P = 0.9$, table 4.1.).

Urinary flow rate revealed a similar pattern. Between pre- and post-heat stress, urinary flow rate fell below the baseline value (table 4.1.). This reduction in flow rate was again more pronounced in EIMD with significant main effects for time and group ($P = 0.001$ and $P = 0.05$, respectively), but no interaction ($P = 0.7$).

Consistent with this, urine specific gravity rose between pre and post-heat stress in both groups but more so in EIMD (table 4.1.). During pre- and post-heat stress, there were significant main effects of time and group ($P = 0.001$ and $P = 0.04$, respectively) but no interaction ($P = 0.2$).

Fractional Excretion of Sodium. Fractional excretion of sodium fell between pre- and post-heat stress but tended to fall more sharply in EIMD (table 4.1.), as evidenced by a main effect of time ($P = 0.01$), and a trend for a time x group interaction ($P = 0.08$).

Muscle Damage and inflammation. As an indirect marker of muscle damage, plasma creatine kinase concentrations at baseline were similar in CON and EIMD (CON: 78 ± 58 IU vs. EIMD: 58 ± 29 IU; $P = 0.1$), suggesting that a sufficient wash out period had occurred. By 24 hours post-treatment, plasma creatine kinase concentrations were significantly elevated in EIMD (CON, 119 ± 69 IU vs. EIMD, 250 ± 69 IU; $P < 0.001$) indicating that the protocol induced some muscle damage. This was corroborated by Delayed-onset muscle soreness scores 24 hours post-treatment (unpublished data). Mean difference (EIMD – CON) and 95% confidence intervals for plasma creatine kinase between both groups were -20 IU [-47, 7 IU] and 131 IU [89, 173 IU] at baseline and post-24 hours, respectively.

Furthermore, at baseline plasma IL-6 was not different between groups ($P = 0.4$; table 4.2). Between pre- and post-heat stress, IL-6 increased in both groups but this rise was greater in EIMD (time x group interaction, $P = 0.005$). In contrast, there were no changes to plasma TNF-α between groups at baseline ($P = 0.8$, table 4.2) or at the pre- to post-heat stress interval (main effect of time $P = 0.6$; main effect of group: $P = 0.5$; interaction: $P = 0.9$).

Exercise intensity. During treatment, both CON and EIMD exercise was conducted at the same exercise intensity with no difference in mean metabolic energy expenditure between treatments (CON: 504 ± 56) W/m^2 vs. EIMD: 503 ± 57 W/m^2; $P = 0.9$). However, during exercise in the heat, mean metabolic energy expenditure was slightly higher in EIMD compared to CON, despite identical running speed and incline (CON: 522 ± 50 W/m^2 vs.

EIMD: 556 ± 57 W/m^2; P < 0.001). Mean difference (EIMD – CON) and 95% confidence intervals for mean metabolic energy expenditure between groups were -1 W/m^2 [-15, 13 W/m^2] and 34 W/m^2 [23, 45 W/m^2] at pre-heat stress and post-heat stress, respectively.

Body temperature and hydration status. Pre and post -heat stress, rectal core temperature was greater in EIMD compared to CON (table 4.2). There was a significant main effect of time and group (P < 0.001 and P = 0.007, respectively), but no interaction (P = 0.2).

Nude body mass decreased between pre- and post-heat stress in both treatment arms (table 4.2). This decrement in body mass was similar for each treatment, hence there was a significant main effect for time but not for group (P < 0.05 and P = 0.8, respectively). Moreover, when body mass change between pre- and post-heat stress was calculated as a proxy for fluid balance there was no difference between EIMD and CON (body mass change EIMD: -0.79 ± 0.37) kg; CON: -0.78 ± 0.16 kg, P = 0.9).

At baseline, plasma volume was estimated as 52.3 ± 3.1 and 51.8 ± 3.0 % of total blood volume, in CON and EIMD groups, respectively. Plasma volume was not different between groups at pre-heat stress and post-heat stress (pre-heat stress: CON, 51.9 ± 3.6 % vs. EIMD: 51.0 ± 3.3 %; post-heat stress: CON, 51.2 ± 3.1 % vs. EIMD, 51.0 ± 3.0 % of total blood volume). No main effects were observed for time (P = 0.6) or group (P = 0.4).

Correlational Analyses. Plasma NGAL was negatively correlated with urine volume in EIMD (r = -0.65; P = 0.04) but not in CON (r = -0.04; P = 0.9), and plasma NGAL was positively correlated with urinary specific gravity in EIMD (r = 0.64; P = 0.05), but not in CON (r = 0.08; P = 0.8). Similarly, a trend existed for plasma NGAL to be correlated negatively with urinary flow rate in EIMD (r = -0.61; P = 0.06), but not in CON (r = -0.05; P = 0.9). Although no associations were noted between plasma IL-6 with markers of kidney function, plasma IL-6 was positively correlated with the biomarker of kidney injury, plasma NGAL, in EIMD (r = 0.88; P = 0.001). Consistent with other measures, this association was not evident in CON (r = -0.06; P = 0.9).

	Group	Baseline	Before heat stress	After heat stress	Before heat stress: EIMD – CON	After heat stress: EIMD – CON
Urine volume (ml)	**EIMD**	**146 ± 105**	**269 ± 133** †	**88 ± 77*** †	**-93 [-253, 66]**	**-81 [-24, -139]**
	CON	**156 ± 96**	**363 ± 175**	**169 ± 78***		
Urine flow rate (ml/min)	**EIMD**	**3.4 ± 2.3**	**3.5 ± 1.9** †	**1.1 ± 1.0*** †	**-1.3 [-3.3, 0.7]**	**-1.0 [-1.7, -0.3]**
	CON	**3.2 ± 1.8**	**4.8 ± 2.2**	**2.0 ± 1.0***		
Urinary specific gravity	**EIMD**	**1.011 ± 0.008**	**1.006 ± 0.004** †	**1.012 ± 0.004*** †	**0.001 [-0.002, 0.046]**	**0.004 [0.001, 0.008]**
	CON	**1.010 ± 0.008**	**1.005 ± 0.004**	**1.008 ± 0.005***		
Fractional excretion of sodium (%)	**EIMD**	**0.39 ± 0.15**	**0.33 ± 0.16**	**0.13 ± 0.12***	**0.04 [-0.08, 0.16]**	**-0.08 [-0.18, 0.01]**
	CON	**0.36 ± 0.12**	**0.29 ± 0.12**	**0.22 ± 0.19***		

Table 4.1. Kidney function markers following exercise induced muscle damage or control exercise. Legend: Data are mean ± SD, and mean difference between groups [95% confidence interval]; EIMD, exercise-induced muscle damage; CON, control; by main effect: *, significantly different from before heat stress; †, significantly different from CON.

	Group	Baseline	Before heat stress	After heat stress	Before heat stress: EIMD – CON	After heat stress: EIMD – CON
Interleukin-6 (pg/ml)	**EIMD**	**0.7 ± 0.5**	**2.8 ± 0.8** †	**6.0 ± 1.9)*** †	**1.4 [0.6, 2.2]**	**3.2 [1.5, 5.0]**
	CON	**0.6 ± 0.3**	**1.4 ± 0.9**	**2.8 ± 1.8)***		
Tumor necrosis factor alpha (pg/ml)	**EIMD**	**1.9 ± 0.2**	**2.0 ± 0.1**	**2.1 ± 0.4)**	**0.06 [-0.13, 0.24]**	**0.07 [-0.23, 0.37]**
	CON	**1.9 ± 0.1**	**2.0 ± 0.2**	**2.0 ± 0.3)**		
Core temperature (^{o}C)	**EIMD**	**37.0 ± 0.3**	**37.8 ± 0.2** †	**39.5 ± 0.5*** †	**0.35 [0.05, 0.65]**	**0.67 [0.13, 1.22]**
	CON	**37.0 ± 0.4**	**37.5 ± 0.3**	**38.8 ± 0.7***		
Nude body mass (kg)	**EIMD**	**70.1 ± 7.7**	**69.2 ± 7.5**	**68.4 ± 7.5***	**-0.03 [-0.25, 0.19]**	**-0.04 [-0.42, 0.33]**
	CON	**69.8 ± 7.3**	**69.2 ± 7.3**	**68.4 ± 7.3***		

Table 4.2. Treatment responses following exercise induced muscle damage or control exercise. Legend: Data are mean ± SD, and mean difference between groups [95% confidence interval]; EIMD, exercise-induced muscle damage; CON, control; by main effect or by *post-hoc* test following significant interaction: *, significantly different from before heat stress; †, significantly different from CON.

4.5. *DISCUSSION*

For the first time it has been shown that prior muscle damaging exercise and its consequent mild inflammatory response led to upregulation of a biomarker of kidney injury, NGAL, when performing exercise in the heat. Prior muscle damaging exercise also resulted in alterations to kidney function as evidenced by increased plasma Cr concentration (by approximately 20%), decreased urine volume and urine flow rate (by 50%), reduced fractional excretion of sodium (by 40%), and mildly increased urinary specific gravity, compared to control exercise. Once AKIN criteria for AKI were applied, half of the individuals in the muscle damage group met stage 1 criteria (plasma Cr > 0.3 mg/dl from baseline) compared to no individuals in the control exercise group. Moreover, the reduced fractional excretion of sodium in the muscle-damaged group suggests increased Na-K-adenosine triphosphatase activity within renal tubules and therefore higher oxygen and energy demands (Katz 1988). These findings reveal that even modest muscle damage, as typically observed following unaccustomed exercise, combined with relatively mild exercise-heat exposure, induces kidney stress.

Such vulnerability of the kidney is of particular relevance to exercise settings and occupations where arduous physical activity is performed in hot conditions. Of note, the functional changes seen in the participants did not represent a positive adaptation to dehydration. These changes occurred despite fluid intake being strictly controlled and matched between groups. Lending further support, hydration status and fluid balance were similar in the muscle-damaged state, as evidenced by near identical body mass and plasma volume changes. The counterbalanced crossover study design also controlled for potential AKI aggravators such as preceding infectious illnesses, dehydration, and use of non-steroidal anti-inflammatory agents (Leon and Helwig, 2010). Taking all of the above into account and noting that the study's population comprised athletic individuals exhibiting a high degree of fitness (mean $\dot{V}O_{2max}$: 59 ml/kg/min), it is plausible that even more profound changes in biomarker and kidney function may result in the field where a "perfect storm" of muscle damage-naïve individuals and multiple environmental stressors are present (Clarkson 2007). Thus, muscle damage should be considered a novel risk factor for AKI.

Changes in plasma NGAL were correlated with altered kidney function (urinary volume, flow rate and specific gravity). Although such data may suggest a maladaptive role for NGAL, it is noteworthy that recent *in-vitro* studies of cell cultures exposed to heat-stress demonstrate that ectopic expression of NGAL may actually have a protective effect

(Roudenkar *et al.*, 2009). The role of inflammatory cytokines also requires further investigation. The increase in both plasma IL-6 and plasma NGAL following muscle damage and the positive correlation between these biomarkers (r = 0.9, P = 0.001 in EIMD group), supports the argument that an acute phase response to inflammation from muscle-damaging exercise may lead to the observed kidney stress. However, a lack of a direct correlation between IL-6 and kidney function does not discount a relationship and may suggest mediation by other factors (Nechemia-Arbely *et al.* 2008; Leon and Helwig, 2010; Lee *et al.*, 2011). Interestingly, TNF-α is unlikely to be a mediating factor due to the lack of change following muscle damaging or control exercise. Alternatively, it is possible that the muscle-damaging protocol used in this study did not produce an IL-6 response sufficient to be directly associated with alterations in kidney function. For example, elevations in plasma IL-6 are often seen in patients with severe sepsis and AKI, but these conditions usually feature concentrations several hundred-fold higher than those observed in the present study (Chawla *et al.*, 2007; Liu *et al.*, 2009; Payen *et al.*, 2012).

Apart from an inflammatory pathway, a reduction in kidney blood flow is another plausible mechanism for the NGAL and kidney function alterations observed. Kidney injury, as indicated by elevations of biomarkers such as NGAL, has been observed in recent clinical studies of "pre-renal" (reduced kidney perfusion) AKI (Nejat *et al.*, 2012). Pre-renal AKI is also known to be associated with heat stroke (Lin *et al.* 2003). Although not directly measured in the present study, the exercise protocol was of sufficient intensity (65% of $\dot{V}O_2$max) to reduce kidney blood flow (Castenfors 1967a; Schrier 1967; Poortmans 1984; Junglee *et al.*, 2012). The observed fall in urine production is also consistent with a reduction in kidney blood flow.

From a practical standpoint, these findings suggest that clinicians should take note of individuals who complain of muscle soreness suggestive of muscle damage either before or after performing strenuous exercise in the heat, as they appear to be at greater risk of developing AKI. In the majority of cases such aberrations will resolve without sequelae. However, if this scenario was amplified and/or occurred as part of a "perfect storm" (as is often the case during heat illness), serious AKI is more likely (Clarkson 2007). Currently, unstandardized protocols exist to check for AKI in the days following exertional rhabdomyolysis-associated muscle damage (Patel *et al.*, 2009). The present results suggest that kidney injury may be induced much earlier. Hence, precautionary measures including serial AKI biomarker sampling may be helpful; particularly given that measurement of

traditional markers of kidney injury such as urine volume can be difficult to ascertain following exercise (Poortmans 1984; Clarkson 2007).

It is recognized that although elevations of urine NGAL with muscle damaging exercise were eight-fold (to 15 ng/ml), with a highest observed value of 65 ng/ml (expected normal range of 0.7-9.8 ng/ml), and elevations of plasma NGAL were two-fold (to 109 ng/ml) with a highest observed value of 158 ng/ml (expected normal range of 37-106 ng/ml), these responses are lower than that used to define clinical AKI (typically 250 ng/ml) (Nechemia-Arbely *et al.*, 2008). It is important to note that it was not a study aim to induce and diagnose AKI. In fact, the interpretation of such sub-clinical elevations is not well-defined at present, but some have suggested they may indicate kidney stress or mild injury (Nickolas *et al.*, 2008; Nejat *et al.*, 2012). In the context of exercise-induced muscle damage, this interpretation is analogous to that of exercise-induced rises in troponin concentrations that have been suggested to be an indicator of mild cardiac stress and, possibly, sub-clinical damage following strenuous endurance activity (Shave *et al.*, 2010).

A key limitation of this study is whether the observed plasma NGAL elevations arose solely from the kidney, as other sources (e.g. respiratory epithelium, liver, heart) cannot be excluded (Chakraborty *et al.*, 2012). Moreover, fresh urine was not microscopically examined for evidence of tubular damage (e.g. for granular casts and for leukocyte quantification), which can also influence urinary NGAL concentrations (Decavele *et al.*, 2011). However, plasma elevations were associated with reductions in measures of kidney function, and urinary flow rates were consistent with increased urinary tubular excretion of NGAL in EIMD. Furthermore, the ELISA kit utilized in this study only detects monomeric urinary NGAL; indicating a predominantly tubular rather than leukocyte origin (Mårtensson *et al.*, 2012). In addition, the current findings are only applicable to a male population. Although the majority of heat stroke and / or acute kidney injury cases following heavy physical exertion are reported in this sex (Carter *et al.*, 2005), it remains to be shown whether females demonstrate similar responses; a sex specific response could be hypothesised given that high oestrogen levels can afford a protective effect against muscle damage e.g. high oestrogen levels (Constatini *et al.*, 2005). Finally, the use of NGAL in this study has provided useful mechanistic information to the evolution of kidney stress during exercise in the heat, but further study is need to determine whether NGAL yields superior sensitivity and / or specificity against more traditional measures such as plasma Cr in such settings.

In conclusion, performing muscle damaging exercise prior to running in the heat results in a mild inflammatory response (as assessed by plasma IL-6), upregulation of a biomarker of

kidney injury suggesting kidney stress (as assessed by NGAL), and alterations to kidney function (as assessed by plasma Cr, urine production and fractional excretion of sodium). Hence, prior muscle damaging exercise should be considered as a novel risk factor for developing AKI when performing exercise in the heat. Further controlled studies are required to dissect other potential interacting risk factors such as prior non-steroidal anti-inflammatory drugs use and intravascular volume depletion. These may have an additive effect with muscle damage and result in clinically significant changes in NGAL concentrations and kidney function measures. This study also raises the intriguing and unanswered question as to whether acclimatizing naïve individuals to muscle damage can ameliorate the consequent inflammation, elevated AKI biomarkers and alterations in kidney function, since pre-conditioning exercise reduces muscle damage through the repeated bout effect (Chen *et al.*, 2012).

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CHAPTER 5

INFUSION OF RECOMBINANT IL-6 IN HEALTHY HUMANS ELEVATES PLASMA NGAL CONCENTRATIONS WITHOUT A REDUCTION IN RENAL FUNCTION

5.1. *ABSTRACT*

Interleukin-6 (IL-6) is believed to be an important cytokine in acute kidney injury (AKI). Whether IL-6 *per se* is able to influence an AKI biomarker (Neutrophil Gelatinase Associated Lipocalin, NGAL) and kidney function in healthy humans, is not known. Thus, a three-hour recombinant IL-6 infusion was administered to six males at a rate of 5 μg/h. Plasma IL-6, NGAL, creatinine (Cr), and cystatin C concentrations were measured at 0 hours, 0.5 hours, 3 hours and post-infusion at 24 hours and 48 hours. Tympanic temperature was recorded at 0 hours and 3 hours. IL-6 concentrations were 0.7 {0.6, 1.2} {interquartile range} pg/ml at 0 hours and peaked at 3 hours to 159.7 {114.6, 186.7} pg/ml. By 48 hours this fell to 8.2 {6.8, 13.0} pg/ml (main effect of time, $P < 0.001$). Plasma NGAL concentrations were 24.3 (13.2) ng/ml at 0 hours and peaked at the end of the infusion at 3 hours to 70.6 (49.3) ng/ml. At 48 hours, this fell to 31.7 (16.5) ng/ml (main effect of time, $P = 0.025$). Plasma Cr and cystatin C concentrations were unchanged. Tympanic temperature rose from 36.9 (0.2) ^{0}C to 37.5 (0.6) ^{0}C at 0 hours and 3 hours, respectively ($P = 0.046$). Plasma NGAL was positively correlated with tympanic temperature ($r = 0.945$, $P = 0.004$). Although IL-6 concentrations found in clinical models of AKI were achieved, elevations in plasma NGAL were below the range typically associated with AKI and no changes to renal function were evident. Overall, these findings suggest that IL-6 is able to modulate NGAL but is not *per se* responsible for AKI or kidney dysfunction, and that addtional physiological aberrations must be involved.

5.2. *INTRODUCTION*

From a histological perspective, acute kidney injury (AKI) is typified by a pro-inflammatory environment (Bonventre and Zuk, 2004; Edelstein and Schrier, 2007). Following an insult that leads to injury of the renal vascular endothelium, there is recruitment of leukocyte subsets such as macrophages and neutrophils from the blood into the kidney interstitium (Friedewald and Rabb, 2004). Together with renal tubular cells, these release various vasoconstrictive mediators and pro-inflammatory cytokines that contribute to the disruption of kidney function through tubular injury and culminates in apoptosis (Edelstein and Schrier, 2003). Thus, cytokines play an important role in both the initiation and extension of inflammation in AKI (Lee *et al.*, 2011).

Interleukin-6 (IL-6) is a cytokine that is thought to play a significant pathogenic role as a mediator of AKI, possibly through trans-signalling and Signal Transducers and Activators of Transcription protein activation in renal tubular cells (Lee *et al.*, 2011). In mouse models of ischemic AKI, there is a rapid but transient increase in IL-6 expression by podocytes (Kielar *et al.*, 2005; Vannay *et al.*, 2009). Also, in direct response to exogenous IL-6, podocytes produce neutrophil gelatinase-associated lipocalin (NGAL); a biomarker of kidney injury (Lee *et al.*, 2012). Despite such findings, the experimental manipulation of IL-6 yields variable responses in different models of AKI. For example in ischemic AKI, the prognosis of IL-6 knock-out mice may not be different or even improved in comparison to wild type mice, but in cisplatin-induced AKI, IL-6 knock-outs fare worse (Patel *et al.*, 2005; Klein *et al.*, 2008; Mitazaki *et al.*, 2011). These disparities could be due to variable doses of insult, experimental time course and the definitions of AKI used, or be reflective of the pleiotropic role of IL-6 as a key immunomodulatory cytokine that is involved throughout the evolution of AKI; from the initial phases of an acute phase response to its reparatory stage and resolution (Hurst *et al.*, 2001; Nechemia-Arbley *et al.*, 2008). Indeed, the modulation of fever by IL-6 through the synthesis of prostaglandin E_2 in the hypothalamus is a well-studied example of its pleotropism (Netea *et al.*, 2000). Mouse models suggest high core body temperatures can moderate severity and recovery of AKI: hyperthermia potentiating injury (Zager and Altschuld 1986). These effects are associated with, and may be influenced by, temperature-induced changes in renal high-energy phosphate availability and oxidant stress during the ischaemic/post-ischaemic period in AKI (Zager *et al.*, 1985).

The above relationships could have importance in a real-world setting. Junglee *et al.* (2013) recently demonstrated that performing muscle damaging exercise prior to running in

the heat increased plasma IL-6 concentration, core body temperature, kidney stress (through a rise in plasma and urine NGAL concentrations), and renal dysfunction (through elevations in plasma creatinine and a fall in urine output) compared to non-muscle damaging exercise. Moreover, plasma IL-6 concentration was positively correlated with plasma NGAL concentration and the latter was correlated with markers of reduced renal function (such as reduced urine production). Thus, it is plausible that IL-6 is part of an acute phase response integral to the development of AKI associated with exertional heat injury. However, whether the release of IL-6 *per se* leads to kidney stress and altered kidney function could not be ascertained from this study, as there could be contributions and interactions from other physiological changes and inflammatory mediators during muscle damage. Additionally, muscle-damaging exercise only elevates IL-6 modestly in laboratory-based studies, whereas much higher IL-6 concentrations are associated with severe AKI (Simmons *et al.*, 2004; Zarjou and Agarwal 2011).

To provide further insight into the role of IL-6 in AKI, the objective of this study was to infuse recombinant IL-6 in healthy participants; allowing us to extend previous animal studies and also to experimentally confirm previous observations of a mechanistic role for IL-6 in AKI (Junglee *et al.*, 2013). For obvious ethical reasons, elevations of plasma IL-6 to concentrations well below those associated with AKI were only sought. Thus, the study's primary aims were to determine the effect of a recombinant IL-6 infusion upon markers of kidney stress (by evaluating plasma NGAL response) and kidney function (by evaluating plasma creatinine (Cr) and cystatin C responses).

Study design and participants. An observational cohort study design was employed. The study was undertaken in accordance with the Declaration of Helsinki (2008) and the institutional ethics committee gave ethical approval. Six healthy Danish Caucasian male participants were recruited (age 25 ± 5 (SD) years; height 181 ± 7 cms; weight 81 ± 10 kg). All provided written consent and none had chronic medical conditions, recent infective illness, heat illness / fever or medication use (e.g. regular use of non-steroidal anti-inflammatory agents) that precluded participation.

Recombinant interleukin-6 infusion. All participants were fasted from midnight prior to receiving a three-hour intravenous infusion of human recombinant IL-6 (Sandoz, Basle, Switzerland). This was delivered in 20% human albumin solution (xenalb20, Bio Products Laboratory, Hertfordshire, UK), at a rate of 5 μg/h in a volume of 25 ml/h, through an 18 gauge venous catheter (BD medical, Albertslund, Denmark) inserted in the antecubital fossa of the non-dominant arm. The group was supervised at all times by a medically trained individual. Similar protocols have been used in previous human studies investigating the effect of IL-6 on cytokine recruitment and general metabolism, and a peak plasma IL-6 concentration similar to that found in post-cardiopulmonary bypass associated-AKI (above 100 pg/ml) can be achieved without significant side-effects (Nemet *et al.*, 2006; Musleh *et al.*, 2009).

Primary and secondary outcome measures. Primary outcome measures were taken during the recombinant IL-6 infusion (at 0 hours, 0.5 hours and 3 hours) and post-IL-6 infusion (at 24 hours and 48 hours; figure 5.1.). These included biomarkers of kidney injury (plasma NGAL) and function (plasma Cr and cystatin C) obtained through sampling of whole blood (K_2EDTA).

Blood samples were centrifuged for 10 minutes at 1500 g and plasma aliquots were stored at -80 ^{o}C for subsequent analysis. To determine if any degree of AKI had occurred, Acute Kidney Injury Network (AKIN) criteria for AKI were used, with any rise in plasma Cr of ≥ 0.3 mg/dl or 1.5 or 2 times higher than baseline indicative of AKI (Ricci *et al.*, 2011). Secondary outcome measures included heart rate (Omron M2, Omron Sante France, Rosny Sous Bois, France), blood pressure (Omron M2, Omron Sante France, Rosny Sous Bois, France) and tympanic temperature (Braun Thermoscan, Fredericksberg, Denmark) that were only recorded prior to and immediately after the infusion had ended (figure 5.1.). The same recorder took all measurements whilst participants rested supine.

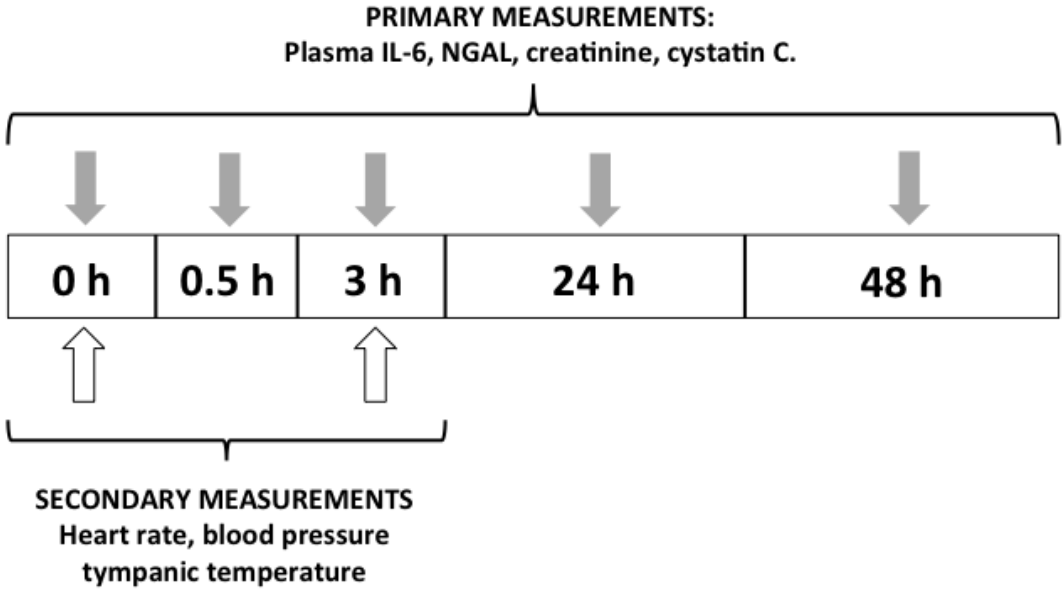

Figure 5.1. Schematic illustrating time points for primary and secondary measurements. The recombinant IL-6 infusion was given between 0 and 3 hours (h). All measurements were taken in the resting condition.

Biochemical Analyses. Enzyme-linked immunosorbent assays (ELISA) were performed as per kit instructions for plasma IL-6 (High sensitivity ELISA kit, R&D systems Minneapolis, MN, USA), and plasma NGAL (NGAL Rapid ELISA kit, Bioporto, Gentofte, Denmark). Absorbances were read by a microplate reader at wavelengths of 450 and 490 nm for NGAL and IL-6 respectively (FLUOstar Omega, BMG Labtech GmbH, Ortenburg, Germany). Plasma Cr was measured by the Jaffe method on an Olympus AU2700 automated analyzer (assay: OSR6178; Olympus, UK; Beckmann-Coulter, UK). Plasma cystatin C was measured by nephelometry on a Roche Integra 400+ analyser (Roche Diagnostic Limited, West Sussex, UK). For each biochemical analysis, all participant samples were assayed on the same plate. Intra-assay coefficient of variation for IL-6, NGAL, Cr and cystatin C were 6%, 5.5%, 5.2% and 0.9%, respectively.

Statistical Analysis. Data for all dependent variables were examined for normality using histogram plots and Shapiro-Wilk tests. Plasma IL-6 and systolic blood pressure were not normally distributed but were successfully transformed using the $\log_{10}$ function for subsequent statistical tests. Thus, mean ± SD are presented for all measures except for IL-6 and systolic blood pressure where median {interquartile range} are given. All time points were compared using parametric one-way repeated measures analysis of variance (time) with adjustments made to the degrees of freedom when assumptions of sphericity were violated.

Planned comparisons were made between specific time points using paired *t*-tests. Paired t-tests were also used to compare outcomes that were measured at 0 hours and 3 hours only (heart rate, blood pressure and temperature).

To determine possible relationships between plasma IL6, plasma NGAL, measures of renal function (plasma Cr and cystatin C), heart rate, blood pressure and body temperature, bivariate Pearson's correlations were performed on raw change scores of values from 0 to 3 hours. SPSS version 18.0 was used for all statistical analyses (SPSS Inc., IBM, Chicago IL, USA) and statistical significance was accepted when $P \leq 0.05$.

For planned comparisons of the primary outcome measure of kidney injury (plasma NGAL) between specific time points, a power calculation revealed that 6 participants were needed to obtain 80% power with a type I error rate of 5% for a two sided paired samples t-test to detect a smallest important change in means of 43 ng/ml (Hopkins 2006). This value is the increase required to elevate plasma NGAL from the normal healthy population mean (63 ng/ml) to above the normal range (37-106 ng/ml). This power calculation used a SD of the difference scores of 10 ng/ml (Junglee *et al.*, 2013).

5.4. *RESULTS*

All six participants completed the study without significant side-effects from infusion of recombinant IL-6.

Infusion of recombinant IL-6. Recombinant IL-6 concentrations were 0.7 {0.6, 1.2} pg/ml at 0 hours and peaked at 3 hours to 159.7 {114.6, 186.7} pg/ml. By 48 hours post-infusion this had reduced to 8.2 {6.8, 13.0} pg/ml (figure 5.2.). Overall, there was a significant effect of time ($P < 0.001$; planned comparisons, 0 to 0.5 hours: $P < 0.001$; 0 to 3 hours: $P < 0.001$; 0 to 24 hours: $P = 0.004$; 0 to 48 hours $P = 0.001$; figure 5.2.).

Plasma NGAL concentrations. Plasma NGAL concentrations were 24.3 ± 13.2 ng/ml at 0 hours and peaked at the end of the infusion at 3 hours to 70.6 ± 49.3 ng/ml, thus representing a mean increase of 167% from baseline (figure 5.3.). By 48 hours, this had fallen to 31.7 ± 16.5 ng/ml (figure 5.3.). There was a significant effect of time ($P = 0.025$; planned comparisons, 0 to 0.5 hours: $P = 0.92$; 0 to 3 hours: $P = 0.027$; 0 to 24 hours: $P = 0.338$; 0 to 48 hours: $P = 0.631$; figure 5.3.).

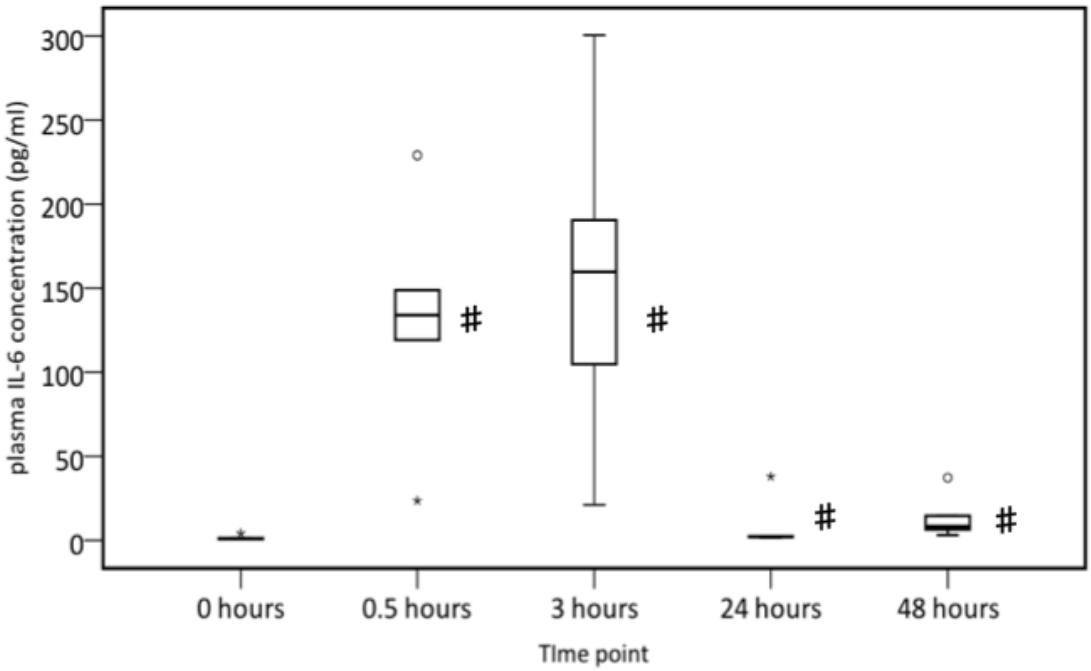

Figure 5.2. Effect of recombinant IL-6 infusion upon plasma IL-6 concentrations. Data are medians (thick lines) and ranges. Open circles and asterixes are outliers. There was a significant effect of time ($P = 0.007$). #, difference compared to 0 hours sample point by planned comparisons.

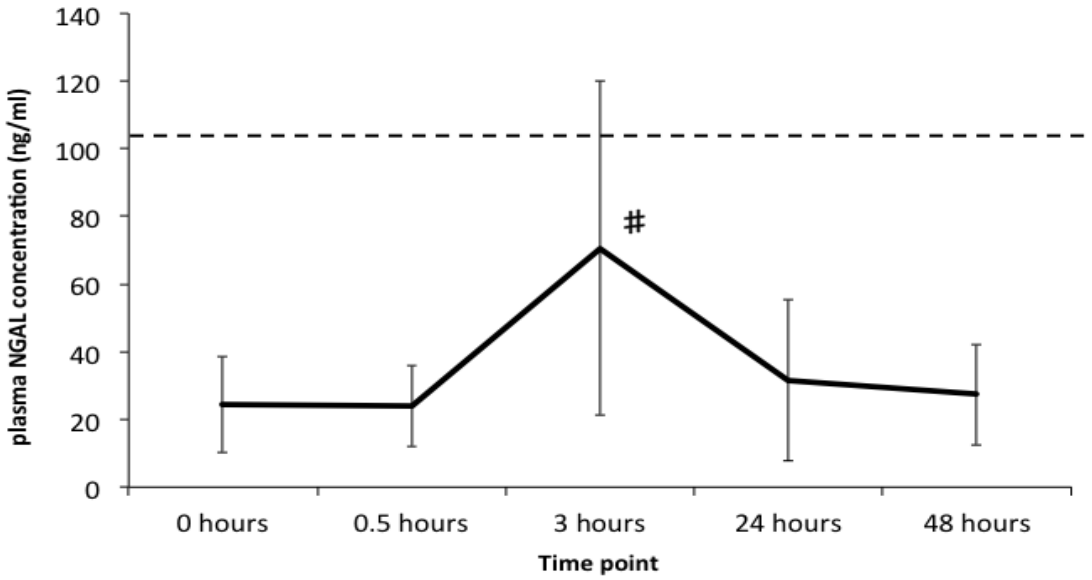

Figure 5.3. Effect of recombinant IL-6 infusion upon plasma NGAL concentrations. Data are means and standard deviations. Dotted line indicates upper limit of normal range for plasma NGAL (106 ng/ml). There was a significant effect of time (P = 0.025). #, difference compared to 0 hours sample point by planned comparisons.

At no time point were mean plasma NGAL concentrations elevated more than the minimum important clinical difference. However, one individual reached a peak plasma NGAL concentration that was greater than the normal range of 37-106 ng/ml (135.5 ng/ml at 3 hours).

Plasma creatinine and cystatin C concentrations. Mean baseline plasma Cr was 0.86 ± 0.28 mg/dl and did not exhibit any significant changes throughout the duration of the experiment (main effect of time: P = 0.465). Furthermore, no subject satisfied any stage of the AKIN criteria. Similarly, mean baseline plasma cystatin C was 0.75 ± 0.14 mg/l and did not change over time (main effect of time, P = 0.176).

Heart rate, blood pressure and tympanic temperature. Heart rate was significantly elevated between 0 and 3 hours of the recombinant IL-6 infusion (P = 0.041; table 5.1.), as was tympanic temperature (P = 0.046; table 5.1.). In contrast, neither systolic nor diastolic blood pressure measurements changed significantly throughout the course of the infusion (P = 0.576 and P = 0.562, respectively; table 5.1.).

Correlational analyses. Plasma NGAL was positively correlated with tympanic temperature (r = 0.945, *P* = 0.004). However, there were no significant correlations between other dependent variables.

Secondary measurement	0 hours	3 hours
Heart rate (bpm)	**61 (7)**	**67 (6)** *
Systolic blood pressure (mmHg)	**120 {3.75}**	**120 {0}**
Diastolic blood pressure (mmHg)	**78 ± 8**	**75 ± 5**
Tympanic temperature (^{0}C)	**36.9 ± 0.2**	**37.5 ± 0.6** *

Table 5.1. Effect of recombinant IL-6 infusion upon heart rate, blood pressure and tympanic temperature at 0 hours and 3 hours. Legend: Data are mean ± SD except for systolic blood pressure (median {interquartile range}). *, significantly different from 0 hours by paired t-test.

5.5. *DISCUSSION*

This is the first study to isolate the relationships between IL-6, a biomarker of kidney injury (NGAL) and established measures of renal function (plasma Cr and cystatin C) in healthy humans. Through a safe infusion of recombinant IL-6, elevations of plasma IL-6 concentrations were demonstrated in six healthy males that peaked by the end of the infusion at 3 hours. There were concomitant elevations in plasma NGAL concentrations, heart rate and tympanic temperature – all of which also peaked at 3 hours. Moreover, plasma NGAL was positively correlated to tympanic temperature. However, although IL-6 appeared to modulate NGAL, mean NGAL concentrations did not exceed the minimum important clinical difference (43 ng/ml) to elevate mean concentrations above normal range (107 ng/ml). Accordingly, with respect to renal function measurements, at a group level there were no changes in plasma Cr and cystatin C throughout the duration of study.

This study yielded plasma IL-6 concentrations close to that found in models of ischaemic AKI from post-cardiopulmonary bypass (Liu *et al.*, 2009; Musleh *et al.*, 2009; Miklaszewska *et al.*, 2013). It is the systemic inflammatory response generated by post-cardiopulmonary bypass that has led investigators to explore the role of humoral mediators in AKI. In a cohort of over one thousand patients who underwent post-cardiopulmonary bypass, the Perioperative Genetics and Safety Outcomes Study group found that an IL-6 polymorphism (-572C) demonstrated a strong association with kidney injury in Caucasian patients ($P < 0.0001$, > 50% decrease in renal filtration when presenting together; Stafford-Smith *et al.*, 2005). However, relevant to plasma IL-6 concentrations achieved in the present study, Musleh *et al.*(2009) revealed that patients undergoing coronary artery bypass grafting had post-operative plasma IL-6 concentrations greater than 100 pg/ml which, in turn, were associated with a risk of kidney dysfunction (defined as creatinine > 176 μmol/l or an increase of 62 μmol/l above the preoperative level; $P < 0.017$, Odds Ratio 1.3 and 95% Confidence Interval 1.0–1.7; Musleh *et al.*, 2009). Other studies have noted much higher increases in plasma IL-6 concentrations associated when severe kidney dysfunction requiring dialysis occurs (Gueret *et al.*, 2009).

In keeping with the aim of the study, the infusion of recombinant IL-6 herein was sufficient to achieve substantial elevations in circulating IL-6 concentrations and up-regulate a biomarker of kidney injury (NGAL). However, the latter was not increased to concentrations found in clinically-defined AKI e.g. > 250 pg/ml (NGAL, Bioporto Diagnostics). Such sub-clinical elevations in plasma NGAL have recently been proposed to

indicate milder degrees of kidney stress or injury such as pre-renal azotemia (Doi *et al.*, 2012; Nejat *et al.*, 2012). Nonetheless, IL-6 failed to impact upon kidney function: neither plasma creatinine nor cystatin C were elevated at intervals of up to 48 hours (Chiou *et al.*, 1975).

The findings from this study may imply that kidney dysfunction is not provoked by IL-6 *per se* and that there are other factors that contribute to its development and AKI (Bonventre and Zuk, 2004; Lee *et al.*, 2011). For example, a critical reduction in renal blood flow in ischaemic AKI is likely to be the initiating factor in establishing a pro-inflammatory environment of numerous cytokines with potentially synergistic activity (Edelstein and Schrier, 2007). This is supported by the post-cardiopulmonary bypass model and in previous work where the effects of muscle damage-induced inflammation followed by exercise in the heat were investigated (Abu-Omar and Ratnatunga, 2006; Liu *et al.*, 2009; Musleh *et al.*, 2009; Miklaszewska *et al.*, 2013; Junglee *et al.*, 2013). In the study by Junglee *et al.* (2013), subjects exercised at intensities where a marked reduction in renal blood flow was expected to occur (65% of maximal aerobic capacity). Thus, despite the comparatively lower endogenous IL-6 response (6.0 pg/ml) observed in that study, a higher mean plasma NGAL concentration (109 pg/ml) was induced that correlated with biomarkers of reduced kidney function (e.g. plasma NGAL and urine volume: r = -0.65; P = 0.04).

Alternatively, when presented without other factors known to induce AKI, the infused IL-6 concentrations may not have been sufficient to induce kidney dysfunction. For example, patients with severe sepsis and AKI harbour endogenous IL-6 concentrations of at least several hundred-fold higher compared to the present study (Simmons *et al.*, 2004; Zaarjou and Agarwal, 2011). Moreover, in its role of treating solid tumours based on its ability of inducing cytotoxic T cell differentiation, recombinant IL-6 infusion rates higher than the present study (150 μg/h) have been responsible for elevations in blood Cr of up to three-times above baseline concentrations in one third of oncology patients (Mulé *et al.*, 1992; Sosman *et al.*, 1997). This observation implies that IL-6 exhibits a dose-related response in its ability to cause renal dysfunction. On the contrary, it is unclear if these previous observations were confounded by factors such as co-morbid disease states (e.g. chronic kidney disease), intercurrent acute illness, and/or the impact of malignancy-related cachexia upon Cr metabolism (Stevens and Levey, 2005). Nevertheless, if extended to the findings of the present study, it is plausible that higher IL-6 concentrations could have resulted in greater plasma NGAL elevations and possibly renal dysfunction.

Aside from being a biomarker of kidney injury, NGAL also plays a role in cytoprotection against adverse environments (Roudkenar *et al.*, 2009; Chakraborty *et al.*, 2012). In one *in-*

vitro study, cell cultures exposed to a temperature of 47^{o}C demonstrated ectopic NGAL expression associated with an anti-apoptotic effect though increased rates of cell proliferation, suggesting that NGAL protected cells from heat toxicity (Roudkenar *et al.*, 2009). The positive correlation of plasma NGAL with tympanic temperature in the present study hints at the potential function of NGAL up-regulation as a cytoprotective factor in conditions of heat stress; in this instance caused by the infusion of recombinant IL-6 (Netea *et al.*, 2000). In AKI, a reno-protective function of NGAL is revealed when administration of exogenous recombinant NGAL in NGAL knock-out mice ameliorates ischemic AKI through inhibition of tubular cell apoptosis (Roudkenar *et al.*, 2009).

A key limitation of this study is the lack of urinary data. Reductions in urinary flow rates are part of current AKIN criteria for AKI (Ricci *et al.*, 2011). Given the possibility that the elevations in plasma NGAL concentrations noted herein could signify a form of very mild AKI that Cr and cystatin C are insensitive to, corroborative evidence from urinary flow rates and other related biomarkers such as urinary NGAL concentrations, fractional excretion of solutes (e.g. sodium) and cytology may have been useful in this regard (Doi *et al.*, 2012; Nejat *et al*., 2012; Perazella and Coca, 2012). However, in animal models where wild-type Sprague-Dawley rats were infused with 50 μg/h of human recombinant IL-6 and achieved plasma IL-6 concentrations of 10-120 pg/ml, there was no microalbuminuria and all had normal renal histology at autopsy (Karkar *et al.*, 1997). Although not entirely comparable to the human model, it does call into question whether any changes to urinary measures would have occurred through the study intervention, especially when plasma NGAL elevations did not exceed the minimum important clinical difference. Additonally, there was no control arm to determine whether a saline or albumin intravenous infusion alone could yield similar elevations in plasma NGAL concentrations. Finally, despite ease of use in a resting state, tympanic temperature readings can demonstrate lower accuracy and precision compared to other measurement sites and may be affected by ambient temperature (Binkley *et al.*, 2002; Lawson *et al.*, 2007). In the study by Junglee *et al.* (2013) rectal temperature was used, which although more invasive, is suited to continuous core body temperature measurement before and during physical activity.

In summary, infusion of recombinant IL-6 in healthy humans that achieves peak plasma concentrations of around 150 pg/ml, leads to sub-clinical elevations of plasma NGAL without impacting upon renal function as measured by plasma Cr or cystatin C. Other factors or higher concentrations of plasma IL-6 could therefore be responsible for AKI. Although speculative, the up-regulation of NGAL through IL-6 and its association with body

temperature suggests a functional role of cytoprotection against heat stressed environments. Finally, the factors responsible for the large inter-individual response to plasma NGAL up-regulation requires further investigation to understand their clinical implications. The genetic makeup of an individual may have some relevance here (Frederickson and Christensen, 2003).

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CHAPTER 6

SUMMARY OF STUDIES, DISCUSSION AND FUTURE DIRECTIONS

6.1. *SUMMARY OF STUDIES*

This PhD sought to explore the acute effects of exercise upon the kidneys to make tenable links to pathological states such as AKI and CKD. This involved experimental *in-vivo* exercise models to simulate a "stressed" kidney. Such associations with their potential clinical implications have received minimal attention despite the increasing number of healthy individuals participating in extreme physical challenges (Draper *et al.*, 2009).

To summarise briefly, **chapter 2** demonstrated that high-intensity exercise in the form of an 800 metre sprint resulted in increased urinary NGAL concentrations and coincident decreases in plasma NGAL concentrations, with both responses being independent from post-exercise proteinuria. Moreover, an inverse relationship was noted between urinary volume and urinary NGAL concentrations; an observation that is also recognised in oliguric AKI.

The systematic review in **Chapter 3** found promise in post-exercise proteinuria as a predictor for CKD progression. Five studies (N = 351) that met inclusion criteria, examined prospective cohorts of Type I diabetics who were at risk of CKD. By combining the results of the primary outcome in four of these studies (N = 318), the presence of PeP was highly associated with elevated resting proteinuria at follow-up (χ^2 test, $P < 0.0001$) and significantly increased likelihood of developing CKD (Odds Ratios: 2.3-52.0). Due to great variability and questionable validity in the interventions performing a meta-analysis was not possible. It was evident that exercise interventions need to be refined and standardised before applying to other at-risk CKD populations.

In **chapter 4**, it was shown that a bout of muscle-damaging exercise established a pro-inflammatory state evidenced by elevated plasma IL-6 concentrations. With subsequent endurance exercise in the heat, increased kidney stress as measured by elevated urinary NGAL and plasma Cr concentrations was discovered. Indeed, plasma Cr was sufficiently elevated to satisfy clincial criteria for AKI. Moreover, plasma IL-6 and plasma NGAL concentrations were positively correlated.

Lastly, **chapter 5** extended the findings of chapter 4 by isolating the role of pro-inflammatory IL-6 in AKI. Through infusion of recombinant IL-6 in healthy males to achieve concentrations above 100 pg/ml, elevations in plasma NGAL were demonstrated but not to concentrations exhibited in AKI. In addition, there were no changes to plasma concentrations of other AKI biomarkers such as Cr or cystatin C. Overall, this suggests that IL-6 is able to modulate NGAL but *per se* is not responsible for AKI or kidney dysfunction. It is likely that additional physiological aberrations are needed.

6.2. *FINDING COMMON GROUND - LINKING STUDY FINDINGS TOGETHER*

6.2.1. *Use of novel kidney injury biomarkers in other studies of physical activity*. How do the findings from chapter 2 and 4 add to the present literature? Chapter 2 represents the only investigation to-date into the response of AKI biomarkers to post-exercise proteinuria elicited by high-intensity exercise (800 metre sprint). Using an endurance-exercise protocol, study 4 demonstrated for the first time that prior muscle damaging exercise followed by additional exercise in the heat, results in upregulation of NGAL and stressed kidney function.

Overall, research in this area appears to be very scarce. At the current time, only two other groups have investigated the response of urine and plasma NGAL to exercise, albeit only in endurance exercise settings. Consistent with the findings from chapter 2 and 4, they have also demonstrated a trend for NGAL up-regulation following exercise.

In a marathon study involving 25 participants, McCullough *et al.* (2011) recorded significant elevations in plasma NGAL (pre-marathon, 8 ± 4 ng/ml; to post-marathon, 47 ± 29 ng/ml; $P < 0.0001$) and kidney injury molecule-1 (pre-marathon, 2.2 ± 1.5 ng/ml; to post-marathon, 3.3 ± 1.7 ng/ml; $P = 0.001$). Similarly, Lippi *et al.* (2012) observed elevations in serum and urine NGAL of 1.6-fold and 7.7-fold, respectively, in 16 participants 20 minutes after completing a 60 km ultramarathon. As in chapter 2, they also noted attenuations in elevations in raw urinary NGAL concentrations when urine NGAL:Cr ratios were compared. Both groups, however, documented post-exercise increases in urinary NGAL greater than chapter 2 (peak median urinary NGAL 13 ng/ml), suggesting that endurance activity may place greater stress on renal physiology. This is somewhat contrary to the commonly accepted view that exercise-induced changes to renal physiology are primarily intensity rather than duration-driven.

It should be noted that in the studies by Mccullough *et al.* (2011) and Lippi *et al.* (2012) there was no quantification of urine output. Therefore, aside from interpreting elevations as renal stress, they could also be viewed as a concentrating effect owing to potentially low-urine outputs from exercise-induced anti-diuresis. Indeed, in chapter 2 that urinary NGAL concentrations were negatively correlated with urine volume ($r = -0.701$, $P = 0.005$). Further study into urinary biomarkers during physical exertion still needs to take these caveats into account to ensure correct interpretation. This is elaborated upon further in the next section.

6.2.2. *Measurement of AKI biomarkers in urine.* In the clinical environment, the measurement and interpretation of urinary AKI biomarkers remains a contentious issue (Waikar *et al*., 2010). Urinary biomarkers can be interpreted as raw absolute values from spot sampling at a single timepoint (Nickolas *et al.*, 2008). However, problems may occur when AKI with a polyuric state is seen and concentrations of biomarker appear to be reduced thus underestimating kidney injury. Conversely, in the more common situation of an AKI with oliguria, concentrations of biomarker may instead appear to be greatly elevated, even if production and excretion rates of the biomarker are constant. If a falling urine flow rate meets the AKI definition, a biomarker measurement in this setting may merely serve to amplify a true-positive result (Waikar *et al*., 2010).

How may such interpretations carry-over into the post-exercise setting? In chapter 2 an inverse correlation between urine volume and absolute urinary NGAL concentrations was observed, which reached its peak at 25 minutes post-exercise. At the same time, urinary A:Cr ratios were also at their highest. Although, the oliguria in this study did not extend to the duration of Acute Kidney Injury Network (AKIN) criteria (e.g. stage 1: < 0.5mg/kg/hr for 6 hours or more), physiologically, this could still be thought of as nephron stress at its peak with increased passage of plasma NGAL and albumin into the urine due to alterations in glomerular permeability. Additional measurement of known biomarkers of renal tubular stress that relate to inhibition or saturation of tubular reabsorption (e.g. β_2-microglobulin, gamma glutamyl-transferase or alpha-1-antitripsin) could lend further support to this notion, and has already received some attention in the post-exercise setting (Ayca *et al.*, 2006; Parikh *et al.*, 2010). Nevertheless, to avoid the issue of a concentrating effect through an oliguric state, there have been attempts to normalise against solutes excreted by the kidney.

6.2.3. *Normalisation against urinary creatinine.* A recognised method of urinary biomarker interpretation involves normalisation against urinary Cr. Apart from its use in proteinuria quantification, urinary Cr normalisation is used to normalise a variety of other urinary analytes due to its ability to correct for changes in urinary dilution. This method relies upon the assumption of steady state excretion of Cr, which is likely in CKD, but is not the case in AKI and possibly the post-exercise setting (Waikar *et al*., 2010). To demonstrate this Tonomura *et al.* examined the influence of AKI induced by various high dose nephrotoxins upon urinary biomarker:Cr ratios using a murine model (Tonomura *et al.*, 2010). By selecting lactate dehydrogenase and N-acetyl-beta-D-glucosaminidase as biomarkers of tubular injury, they discovered that correction by Cr could overestimate AKI, but that receiver-operator

curve analysis suggested this method exhibited a higher diagnostic power than correction by urine flow rates. In addition, where there was a constant urinary biomarker excretion, a decrease in urinary Cr clearance led to an increase in the biomarker:Cr ratio. They concluded that when using Cr correction the direction of alteration of the urinary biomarker concentrations and Cr should be considered. Goldstein has summarized this schematically (figure 6.1.).

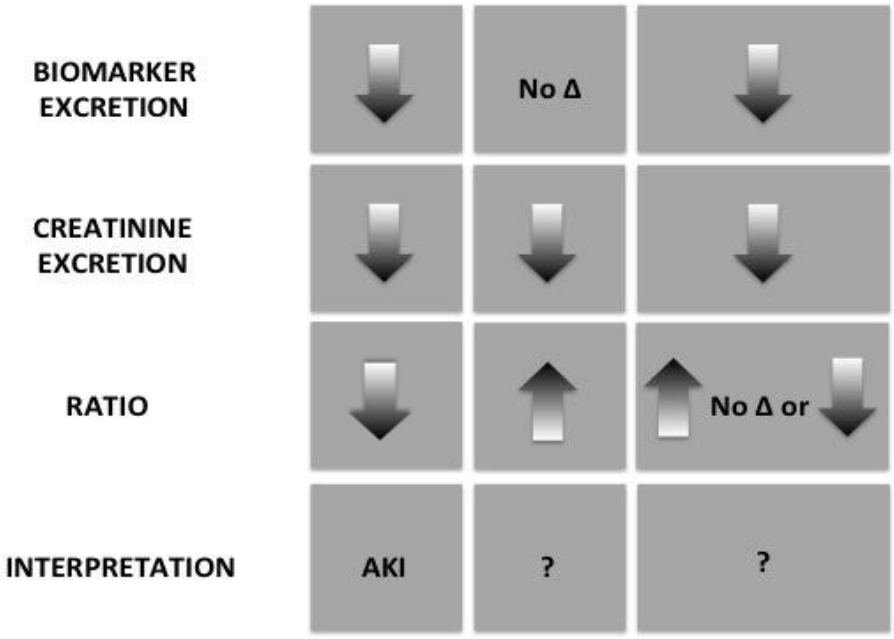

Figure 6.1. When considering urinary biomarker:creatinine ratios, the change of excretion of both biomarker and creatinine should be known. As creatinine excretion is unstable in AKI, changes in the ratio cannot be determined unless the directional change of each is known. This would also apply to other dynamic setting such as post-exercise. AKI, acute kidney injury; Δ, change. Adapted from Goldstein 2010.

In human studies such as those in chapter 2 and by Lippi *et al.* (2012), normalisation of urinary NGAL against urinary Cr appeared to attenuate rises compared to absolute urinary NGAL concentrations, although some of the subjects from the study in chapter 2 continued to show substantial rises in urinary NGAL:Cr. Taking these considerations into account, interpreting Cr normalisation in exercise settings could underestimate the degree of stress placed upon the kidney. Overall, these observations at least indicate some caution should be taken when using urinary Cr normalisation and should prompt further work in glomerular and tubular handling characteristics during dynamic instances. This could be refined further by

incorporating the clinical variables known to affect Cr metabolism such as age, weight, sex, and ethnicity.

6.2.4. *Normalisation against urinary flow rates.* Normalizing to the observed urinary flow rate at the time of urine sample collection to determine a biomarker excretion rate, or performing several timed collections of urinary biomarkers measurements could avoid some of the issues experienced through urinary Cr normalization. In chapter 4, absolute urinary NGAL concentrations were corrected for urinary flow rates. Between pre- and post-heat stress, non-muscle damaged individuals demonstrated a fall in urinary NGAL flow rate whereas muscle damaged individuals exhibited a slight rise (consistent with increased kidney stress during physical exertion in heat and muscle damage).

The incorporation of urinary flow rate into NGAL measurements could be viewed as a more robust interpretation of biomarker values, as urine output is a well-established and validated biomarker of AKI (Ricci *et al*., 2007). Although achievable in the clinical setting from a recumbent patient with AKI or an experimental setting, how this may be accomplished in a real-world setting during physical activity is challenging and will involve consideration of logistic issues surrounding (multiple) sample collection and exercise-induced anti-diuresis.

6.2.5. *Normalisation against urinary osmolality and specific gravity.* Correction of solutes based on urinary specific gravity or osmolality has been investigated in non-AKI settings. However, in AKI these two parameters undergo complex alterations due to renal tubular dysfunction with simultaneous reductions to GFR. This includes urinary dilution and altered concentrations of solutes such as glucose, sodium and potassium which appears to be more complex than urinary Cr handling. In short-term acute exercise of moderate intensity there is an increase in urine osmolality of around 40 mOsmols/KgH_20, whilst at higher intensity exercise further decreases in tubular function may result (Freund *et al.*, 1991). Therefore, it is essential that such dynamic interactions are probed in detail before deciding if these forms of adjustment can be helpful in the interpretation of biomarker concentrations in dynamic circumstances.

6.2.6. *A general interpretation of biomarker elevations during exercise.* Regardless of the above considerations, when the findings in chapters 2, 4 and the other highlighted studies are interpreted together, it is reasonable to conclude that there is a common suggestion of mild kidney injury or stress that occurs during heavy exercise.

The Bioporto NGAL Rapid ELISA Kit used in the experiments herein considers a NGAL concentration cut-off of 250 ng/ml (in urine or plasma) to indicate the presence of a renal disorder, including AKI, without incurring the risk of an unacceptably high proportion of false positive diagnoses (NGAL Rapid ELISA, Bioporto, Gentofte, Denmark). The NGAL concentrations achieved in both urine and plasma in the controlled laboratory-based studies of this PhD could therefore be considered as within a sub-clinical range. Currently, interpretation at this level remains a grey area, although recent evidence suggests that this may represent a mild degree of kidney injury that equates to pre-renal AKI. In a study by Nejat *et al.* (2012), individuals with pre-renal AKI (defined as AKI that recovered within 48 hours, and with a fractional excretion of sodium of less than 1%) had greater median concentrations of several AKI biomarkers compared with no-AKI. Furthermore, Seibert *et al.* (2013) determined that urinary NGAL concentrations in pre-renal AKI patients (as defined by rapid response of renal function to volume repletion), were significantly higher than in healthy controls (pre-renal AKI: 64.8 ± 62.1 ng/ml; controls: 14.4 ± 8.3 ng/ml, $P = 0.001$). Hence, considering that the studies of this PhD involved healthy disease-free individuals and were performed in controlled experimental settings that did not set out to induce clinically-defined AKI, it is plausible that mild pre-kidney injury through tissue hypoperfusion accounts for the results found, as heavy intensity-based exercise is capable of reducing RBF up to 50% from baseline values (Walker *et al.*, 1994).

The contention that mild kidney injury occurs during heavy exercise is also is supported by two further observations from the experiments in chapter 4: 1) based on post-exercise serum creatinine measurements in chapter 4, McCullough *et al*. (2011) and Lippi *et al.* (2012), stage 1 AKIN criteria for AKI were met for at least 40% of participants in each study; and 2) there were concomitant changes in traditional urine physiology measurements such as reduced urine output / flow rate and increased specific gravity, implying stress in renal tubular transport. Together with measurement of novel biomarkers, this latter finding supports the idea of site-specific nephron stresses.

6.2.7. *Post-exercise proteinuria, NGAL and locations of nephron stress*. The measurements of renal biomarkers in chapter 2 and 4 have provided insight into site-specific renal stress that occurs during exercise. It was originally hypothesised in chapter 2 that individuals who had higher concentrations of post-exercise proteinuria would also experience greater levels of renal stress as measured by an AKI biomarker. Although this hypothesis was not supported, as evidenced by the lack of a positive correlation between post-exercise proteinuria and

urinary NGAL concentrations, such discordance could instead reflect a difference in the locations of nephron stress i.e. urinary NGAL arising from tubules and albumin from glomerular leak. Indeed, *in-vivo* and *in-vitro* studies have clearly demonstrated that the vast majority of NGAL up-regulation in models of pre-renal AKI occurs within the proximal and distal tubules (figure 6.2.; Mishra *et al.*, 2003).

This observation is further supported by the findings from Chapter 4 that relate NGAL to altered renal tubular mechanisms during exercise. Here, there were higher concentrations of urine and plasma NGAL in the muscle-damaged group following heat stress with concomitant fall and rise in urine volume and specific gravity, respectively. Moreover, in the same group, plasma NGAL was negatively correlated with urine volume (r = -0.65; P = 0.04), positively correlated with urinary specific gravity in EIMD (r = 0.64; P = 0.05), and tended to be correlated negatively with urinary flow rate (r = -0.61; P = 0.06).

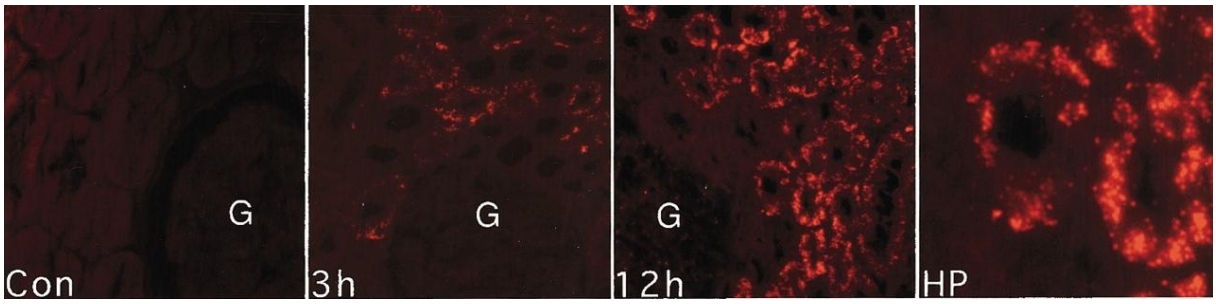

Figure 6.2. Immunohistochemistry results on frozen sections of mouse kidneys at 3 and 12 hour reflow periods following experimentally-induced ischaemia using a vascular clamp. Induction of mouse kidney NGAL protein within tubules is demonstrated by cherry-red fluorescence when tissue samples were probed with a polyclonal antibody to NGAL. Con, sample obtained from control mice; G, glomerulus; HP, sample at 100× magnification (other panels are at 20× magnification). Adopted from Mishra *et al.*, 2003.

Another possible reason for discordance between albuminuria and urinary NGAL elevations is that passage of albumin does not reflect nephron injury (i.e. glomerular basement membrane stress) in healthy people during an acute bout of exercise, but is merely represents a function of momentarily increased glomerular permeability. This is somewhat contrary to the situation in CKD where longstanding glomerular passage of albumin will eventually contribute to nephron injury, as protein overload in the renal tubules induces the release of cytokines, chemokines and growth factors (Abbate *et al.* 2006). This leads to abnormal interstitial accumulation of inflammatory cells, extracellular matrix collagen, fibronectin and other components that culminate in interstitial fibrosis, a pathological hallmark of CKD. Indeed, individuals with CKD show greater exercise-induced declines in GFR compared to

healthy controls and that post-exercise proteinuria may be exacerbated by exercise (e.g. in the nephrotic syndrome) (Bellingheri *et al.* 2008). Despite such findings, regular exercise in CKD does not appear to accelerate disease progression. In fact, exercise programs form an integral part of CKD management strategies.

But how are the findings in chapter 3 accounted for? Here, acute exercise albuminuria was predictive of developing CKD in type 1 diabetics without nephropathy at baseline, with odds ratios between 2.3-52.0. Previous work showed that exhaustive exercise in type 1 diabetics did not appear to provoke enhanced acute renal dysfunction compared to healthy controls, even when there was evidence of other diabetic complications such as retinopathy (Mauer *et al.* 1997). It could be that post-exercise proteinuria in this population instead indicates the development of subtle pathology over time which is specific to diabetics e.g. generalized endothelial dysfunction, which heralds the development of CKD.

6.2.8. *Evidence for renal inflammation during heavy physical activity.* Chapter 4 explored IL-6 as an inflammatory component of renal stress during heavy physical exertion. This was though a muscle-damaging protocol that, as previously demonstrated, successfully yielded supra-normal concentrations of pro-inflammatory IL-6, e.g. Nybo *et al.* (2002) performed an interventional study involving exercise in the heat and achieved IL-6 elevations with a pattern comparable to interventions in chapter 4 (figure 6.3.).

The quantity of IL-6 release appears to be affected by both exercise intensity and duration (Mendham *et al.*, 2011). Also, the type of exercise is relevant as eccentric (muscle damaging) contractions elicit a more inflammatory response, and hence greater increases in IL-6, than concentric contractions. Thus, in recent years, IL-6 has been coined a myokine (a cytokine secreted by and acting upon active muscle) and though several sources of IL-6 have been shown, contracting muscles contributes to most of the circulating IL-6 in response to exercise (Muñoz-Cánoves *et al.*, 2013).

Acute kidney injury is considered an inflammatory disorder and histologically this is evidenced by leukocyte recruitment and tubular injury. This environment is cytokine-rich and such molecules are believed to be important components of both the initiation and extension of kidney injury (Lee *et al.*, 2012). In ischaemic AKI models, many pro-inflammatory cytokines / chemokines are found, including: IL-2, IL-6, IL-10, IL-18, CXCL1, granulocyte-macrophage colony-stimulating factor, tumor growth factor-β, macrophage inflammatory protein-1, and monocyte chemoattractant protein-1 (Lee *et al.*, 2011).

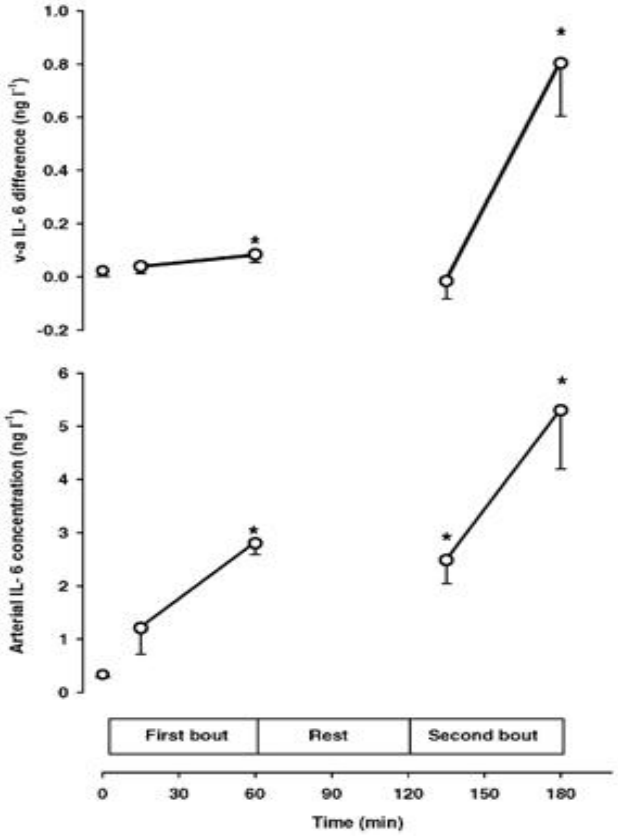

Figure 6.3. Arterial IL-6 concentrations at rest and during exercise during one hour on a cycle ergometer at a core temperature of 38^0C (first bout) followed by a one hour rest period and then further exercise at 39.5^0C (second bout). Both exercise trials were completed at a workload (170 ± 4 W) that corresponded to 50 % $\dot{V}O_{2max}$. A similar pattern of plasma IL-6 rise was also seen in study 4. Data are mean values of 8 subjects. * Significantly different from the resting value ($P < 0.05$). Adopted from Nybo *et al.* 2002.

In chapter 4, the muscle-damaged group had higher plasma IL-6 concentrations and also exhibited greater levels of kidney stress (e.g. reduced urine flow and increased urinary specific gravity). These observations are supported by work from Sugama *et al.* (2013), who demonstrated significant raw and urinary flow-rate corrected elevations in urinary IL-6 in 14 male triathletes following completion of a three-hour dualthon. They also showed similar elevations in other AKI-related cytokines (e.g. IL-2, IL-10 and interferon-γ) in the urine. In addition, and complementary to the findings of chapter 4, they applied AKIN AKI criteria to their cohort and found that "damaged individuals" (i.e. those who exhibited granular casts and those of renal tubular cells and therefore suggestive of renal injury) met stage I immediately, and 90 minutes, post-exercise based upon serum Cr concentration rises (increase ≥ 26 μmol/l within 48hrs or increase ≥ x 1.5 to x 1.9 of baseline serum Cr; figure 6.4.; Sugama *et al.* 2013).

Interpreting these findings together suggests that during heavy exercise, the kidney (along with the active musculature) contributes to IL-6 synthesis and release, and plays a role in kidney stress or mild kidney injury. Indeed, urinary IL-6 has already been proposed as a biomarker of AKI in children undergoing cardiopulmonary-bypass surgery (Dennen *et al.*, 2010). However, can IL-6 by itself participate in kidney stress during strenuous physical activity? The following section will discuss this further.

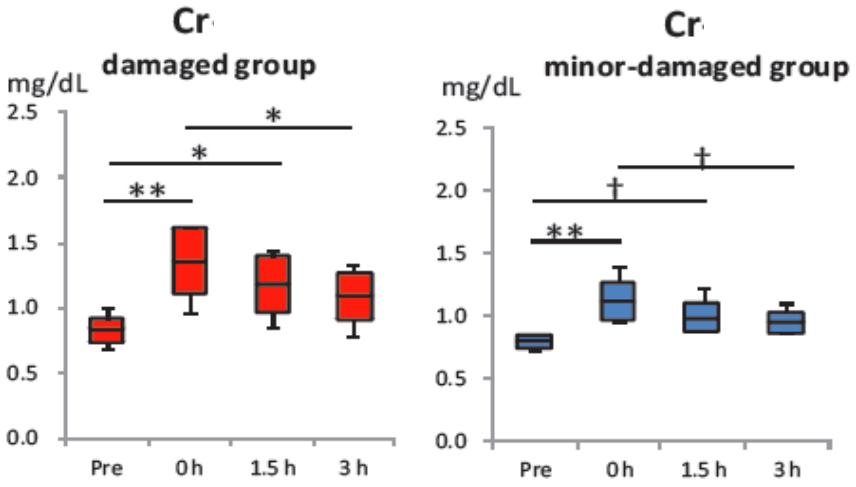

Figure 6.4. Changes in serum creatinine in fourteen male athletes before and at several time points following a 50km dualthon. The cohort was split into a damaged group (N = 7) consisting of individuals with renal tubular epithelium in their urine and a non-damaged group (N = 7) where this was absent. Damaged individuals met stage I AKIN AKI criteria immediately post-exercise and 90 minutes post-exercise based upon serum creatinine concentrations elevations (0.54 mg/dl or 1.66-fold increase from baseline and 0.36 mg/dl or 1.44-fold rise from baseline, respectively). Legend: creatinine (Cr), pre-exercise (Pre), immediately post-exercise (0 h), 1.5 hour post-exercise (1.5 h) and 3 hour post-exercise (3 h) are sampling points. Statistics: * $P < 0.05$, ** $P < 0.01$, † $P < 0.1$. Adopted from Sugama *et al.* 2013.

6.2.9. *Can IL-6 per se lead to kidney injury?* Given the evidence in the literature of IL-6 participating in the evolution of AKI and the results of chapter 4 that showed a strong positive correlation between plasma IL-6 and an AKI biomarker (NGAL), chapter 5 investigated the propensity of IL-6 *per se* to cause kidney injury. To summarise, a recombinant IL-6 infusion at 5 µg/h in healthy individuals was able to up-regulate plasma NGAL but only to sub-clinical concentrations and without changes to plasma cr or cystatin C.

There are a few plausable explanations for such outcomes. It could be that supraphysiological IL-6 concentrations result in a sub-clinical renal (tubular) injury, as indicated by elevated plasma NGAL but not reflected by filtration biomarkers. As discussed

above, recent evidence suggests that in the setting of kidney injury lower concentrations of NGAL (e.g. less than 250 ng/ml) may represent a milder injury that equates to pre-renal AKI (Seibert *et al.*, 2013). Future studies should clarify this with concomitant urinary measures of renal injury. Alternatively, it could be that IL-6 merely modulates NGAL without sequalae.

However, infusions of IL-6 at a higher rate of 150 μg/h have been known to cause kidney injury (as defined by elevated blood Cr concentrations) when used to treat oncology patients with head and neck malignancy (Mulé *et al.*, 1992; Sosman *et al.*, 1997). Similarly, interleukin-2 (IL-2), another AKI-related cytokine, exhibits dose-limiting renal toxicity when used in the treatment of metastatic renal cell carcinoma and metastatic melanoma. More than 90% of patients treated with intermediate or high-dose IL-2 showed some degree of acute renal dysfunction, inducing oliguria in over 60% of patients and grade 4 oliguria / anuria (defined as life-threatening consequences of kidney injury and/or dialysis indicated) in 10% (Poust *et al.,* 2013). It should be noted that in these clinical setting, individuals had disease processes that could potentially sensitize them to the side-effects of cytokine therapies. Nevertheless, these observations imply that interleukin infusions possess a dose-response relationship and higher infusion rates could result in more profound elevations of plasma NGAL with simulatanous derangements to traditional kidney function measures.

What could be the mechanism of cytokine-related renal injury? Interleukin-2 leads to intrarenal vasoconstriction, thought to be a result of abnormal renal prostaglandin synthesis. This, together with a vascular leak syndrome, is likely to contribute to acute tubular necrosis (Poust *et al.,* 2013). Aside from individual cytokine-specific effects, high-dose interleukin infusions in general can lead to a rapid recruitment of numerous cytokines ("cytokine storm"), and the positive feedback reaction between cytokines and leukocytes establishes an inflammatory environment that stimulates AKI (Panelli *et al.*, 2004; Otto *et al.*, 2013). Therefore, despite the perceived importance of IL-6, it is more likely that an accumulation of numerous pro-inflammatory mediators rather than one particular cytokine contributes to AKI.

6.3. *METHODS OF ASSESSING KIDNEY FUNCTION AND KIDNEY INJURY IN EXERCISE – CURRENT STATUS AND DEVELOPMENTS*

6.3.1. *Measuring glomerular filtration rate during physical activity.* A gold standard of GFR estimation involves the intravenous injection of an inert polysaccharide such as inulin or a contrast agent that possesses minimal renal tubular reabsorption and secretion and is, therefore, largely reflective of glomerular filtration. This could be considered an invasive method to determine GFR as it involves intravenous infusions which may be continuous and blood sampling at multiple time points to determine the rate of agent elimination from the blood.

Although practically feasible in the sedentary subject, to make these assessments in individuals during dynamic situations such as exercise is more challenging. Consequently, there has been a trend in physiological studies to use equation-based estimated GFRs that do not require administration and sampling of an agent but only age, sex and a spot blood Cr measurement. Although convenient, such formulae have been principally developed in populations with CKD and should be used with caution in subjects having atypical anthropometric characteristics e.g. rugby players (Banfi *et al.*, 2009). Also, the equations assume stable Cr metabolism (i.e. relatively constant rates of production and excretion) and this is unlikely when there is an abrupt change to GFR such as with exercise. Regardless of these drawbacks, this method continues to be widely used.

Recently, a promising novel and more rapid method of real-time GFR estimation has been described using transcutaneously measured elimination kinetics of a fluorescent polymer (FITC-sinistrin), paired with a low-cost portable sensor that transcutaneously excites FITC-sinistrin at 480 nm and detects the emitted light through the skin at 520 nm (Schreiber *et al*. 2012). Radio-frequency transmission of data allows remote monitoring and real-time analysis of FITC-sinistrin excretion as a marker of GFR. Although only currently developed on murine models, this method has the potential of being an attractive, less invasive alternative for real-time GFR estimation that could be adapted to exercising individuals in a laboratory environment. As a proof-of-principle, comparative measurements of transcutaneous and plasma elimination kinetics of FITC-sinistrin demonstrated good agreement in a various murine models of kidney disease (Schreiber *et al*. 2012).

6.3.2. *Measurement of urine output during exercise studies.* In the emergency medical setting, measurement of urine production represents the most practical real-time measure of kidney function and features heavily in guidance for the management of AKI (cf. AKIN criteria for AKI). Nevertheless, measurement may not be straightforward for a number of reasons, including: lack of urine production, barriers to urinary tract catheterisation, poor recording of output. Even within the context of a controlled laboratory-based environment, measurement of urine output during exercise studies in humans can be challenging. This is important to consider for future studies into renal injury and function during exercise, as: 1) urine output is considered as a biomarker of kidney injury; and 2) accurate collection is important for subsequent determination of urinary biomarker concentrations and / or flow rates.

Early physiological experiments conducted from the 1950s to 1970s commonly inserted urinary catheters *per urethra* into the bladder of subjects to facilitate real-time urine volume and rate calculation (Castenfors 1967a, Castenfors 1967b). Such a procedure could be considered as a "gold standard" for urinary volume or rate assessments, but in both males and females, insertion is not without risk as potential complications include infection, bleeding, urethral damage / scarring, and bladder spasm (Dellimore *et al.*, 2013). This is in addition to the discomfort caused by catheter insertion that may in turn lead to less-than-optimal performance during intensity or duration-based exercise interventions. Such reasons, taken together with increasing ethical scrutiny for research in healthy humans, have made this technique less popular in mpre recent times (Resnik 2012).

In the studies described in chapters 2 and 4, double voiding techniques were employed to maximise bladder emptying and obtain urine. However, residual urine volumes that were not excreted could potentially have carried-over into the next sampling time point. Thus, over- or underestimation of urine volume and flow rates may have occurred and confound biomarker excretion rates. A potential non-invasive method to resolve this issue is to perform bladder ultrasound scans that can provide an accurate estimation of urine volume within the bladder. Volume estimations computed by the Bardscan system correlate closely with the bladder volumes detected on urinary catheterization ($r = 0.982$, $P = 0.016$) (Abdel-Fattah and Barrington, 2005). Different portable scanners are available that require very little training to use, but are not equally accurate nor provide the same level of clinical agreement. The accuracy and level of clinical agreement appears to be greatest when using a 3-D ultrasound system to calculate the bladder volume (Ghani *et al.*, 2008).

From a physiological perspective the magnitude, duration and rate of urine output during exercise are not readily predictable. Rising ADH concentrations are responsible for this fall in

urine output and once higher exercise intensities are approached there is a decrease in free-water clearance. Even hyperhydration, prior to or during severe exercise, does not prevent the decrease in urine flow post-exercise (Refsum and Strömme 1975; Virvidakis *et al*., 1986). Although this cannot be avoided, it seems sensible to ensure that participants are well-hydrated before performing strenuous physical activity. An acceptable limit of hydration can be assessed using a portable osmometer to check urine osmolality immediately prior to exercise, with a cut-off of less than 700 mOsmols/KgH_20 being acceptable of an adequate hydration status (Sawka *et al.*, 2007).

6.3.3. *Use of other molecular biomarkers of kidney injury during exercise.* Of the numerous AKI molecular biomarkers available for clinical and research purposes, NGAL was chosen for use in this PhD as it has a rapidly growing evidence-base and is currently regarded as the most promising for routine clinical use. Irrespective of issues pertaining to interpretation, time of sampling and confounding factors (which are also common to other AKI biomarkers), to-date, all clinical NGAL studies combined include more than 7000 patients (Hasse-Fielitz *et al.*, 2014).

As mentioned previously, the few studies investigating kidney injury during physical exertion have also chosen NGAL as a primary outcome measure. In their marathon cohort of 25 athletes, McCullogh *et al.* (2011) also measured cystatin C and kidney-injury molecule 1. Although not strictly an AKI molecule, plasma cystatin C in this study exhibited parallel and similar mean concentration rises to plasma Cr. Kidney injury molecule-1 (a transmembrane protein that is specifically up-regulated in dedifferentiated proximal tubule cells after ischaemic or nephrotoxic AKI), was also increased during the same period (baseline: 2.6 ± 1.6 ng/ml; immediately post-race: 3.5 ± 1.6 ng/ml, $P = 0.001$; McCullogh *et al.*, 2011).

Measurement of an AKI biomarker panel may therefore serve to improve the sensitivity and specificity of AKI diagnosis, provide more information on site-specific kidney stresses and prognostication. However, it should be remembered that release kinetics of different biomarkers vary, which may influence their application. For example, in clinical studies, large and significant increases of kidney injury molecule-1 concentrations tend to occur 12 hours post-insult, whereas NGAL can be up-regulated as early as two hours from the time of insult (Mishra *et al.*, 2003). The modest kidney injury molecule-1 elevation immediately post-marathon in the McCullough *et al.* (2011) study was made at a mean time of four hours, and so it could be perceived that assessment of this biomarker immediately following shorter duration intensity-based activity may not be sensitive enough to detect kidney stress.

Therefore, in addition to considering how molecular biomarker measurements should be interpreted (see section 6.2.), it is also likely that biomarker panels have to be tailored to the physical activity being investigated.

It has been suggested that cystatin C represents a more precise measure of kidney disease compared to creatinine and should replace the latter as a biomarker of AKI, especially as cystatin C concentrations are less influenced by age, sex, race and muscle mass. Regardless of this, altered cystatin C concentrations (like increased albuminuria) in AKI can only be viewed as a function of glomerular permeability and do not directly indicate increased glomerular basement membrane stress or injury. Animal models of diabetic nephropathy have hinted at new biomarkers of glomerular injury that focus upon actual disruption of basement membrane elements responsible for the filtration process e.g. type IV collagen and laminin (Matheson *et al.*, 2010). The urinary excretion of type IV collagen has been associated with glomerular injury and correlates with the urinary excretion of other components of the glomerular basement membrane including laminin (Yazawa *et al.*, 2002). Further work is needed to define their role within clinical settings, but there is potential to refine AKI diagnosis alongside markers of tubular injury. However, it should be borne in mind that in the context of a post-exercise setting, such components of the glomerular basement membrane are also shared by muscle tissue breakdown and may therefore confound interpretation when there is suspicion of renal injury.

6.4. *FUTURE DIRECTIONS AND CLINICAL IMPLICATIONS OF STUDY FINDINGS.*

6.4.1. *The initiator of renal stress - determination of renal blood flow during physical activity.*

Although the last five to ten years has seen a dramatic rise in AKI molecular biomarker studies, there are still teething issues that are hampering their widespread use. In the clinical environment, their performance is driven by patient characteristics, co-morbid illness, the inciting kidney injury event, timings of measurement and selected thresholds for diagnosis. In this PhD, their application has provided greater insights into the mechanisms of renal stress and injury during heavy physical exertion. Nonetheless, there have been efforts to seek other physiological biomarkers that may indicate AKI and its earliest manifestations which could also be applied to exercise research.

As discussed in chapter 1, heavy exercise results in incremental but pronounced reductions to renal blood flow. This is to the extent that vigorous activity (≥ 80% $\dot{V}O_{2max}$) can result in renal plasma flow decreasing to 50% from baseline (Walker *et al.*, 1994). This change to renal blood flow during exercise is likely to be the inciting stressor that contributes to subsequent alterations in renal physiology i.e. haematuria and proteinuria. Thus, it seems logical that pathophysiological changes to the renal microcirculation occur before cellular injury results in up-regulation of molecular biomarkers of AKI, such as NGAL. Renal blood flow quantification as a biomarker for acute renal injury is technically challenging - even in stationary patients and although surrogates such as renal plasma flow (chapter 1.1) can be assessed, this is resource dependent and time-consuming. Recently, several novel methods for assessing renal blood flow have been described and hold promise for future exercise study.

6.4.1.1. *Doppler ultrasound.* Doppler ultrasound has so far received little consideration as a marker of RBF during exercise; mainly owing to a number of technical issues. Nonetheless, there are other surrogate measures of RBF that may be derived from Doppler and warrant further investigation into their applicability during exercise. These include the renal resistive index and gas-filled microbubble contrast-enhancement. The resistive index ((peak systolic velocity − end diastolic velocity) / peak systolic velocity)), assesses renal vascular resistance and a value of 0.70 is considered to be the upper threshold of normal resistive index in adults. In a study by Lerolle *et al.* (2006) involving a small cohort of patients with septic shock, the resistive index predicted the development of AKI five days after presentation (resistive index of 0.77 vs. 0.68 in AKI vs. non-AKI, respectively). Moreover, the resistive index was found

to be higher in patients with acute tubular necrosis (0.85) than in pre-renal AKI (0.67), and better than urinary biomarkers in the diagnosis of established AKI (Platt *et al.*, 1991).

Contrast-enhancement Doppler through intravenous injection of gas-filled microbubble particles is able to provide information of blood flow at the level of the capillary bed. Its use is well-established in the assessment of myocardial perfusion during echocardiography. In the kidney, there is good correlation between this methodology and RBF as determined by p-aminohippuric acid clearances in porcine and canine models. In a prospective cohort trial, Kalantarinia *et al.* (2009b) showed that following a renal stressor in the form of a high-protein meal (1.5 g of chicken / kg body weight), there was a 42% increase in cortical blood flow and a 37% increase in blood velocity by using this technique. The authors also concluded that this technique was rapid, safe with a low rate of mild adverse events (about 5%).

In terms of practicality, ultrasound offers portability and rapid result turnover but is heavily dependent on operator experience. Some of these characteristics are desirable if the above measures were to be used in the field to determine AKI risk in heavy physical activity. For example, assessing RBF in this manner may allow quantification of a "renal reserve" and provide an *a priori* estimation of AKI risk.

6.4.1.2. *Magnetic resonance imaging*. Magnetic resonance imaging (MRI) may also be used to assess parenchymal renal blood flow. Blood Oxygen Level-Dependent MRI is a rapid and non-invasive method that relies upon paramagnetism to detect deoxyhaemaglobin that increases in proportion to oxygen consumption in renal tissues. For example, in a murine study, Juillard *et al.* (2004) demonstrated that this variation of MRI could noninvasively detect change in intra-renal oxygenation during an acute reduction of RBF due to unilateral renal artery stenosis.

Application in kidney exercise physiology models also appears feasible. Using a custom MR-compatible cycloergometer, Taylor *et al.* (2002) revealed that blood flow to the digestive and renal circulations decreased from 2.1 +/- 0.5 l/min at rest to 1.6 +/- 0.7 l/min during exercise ($P < 0.01$), while infrarenal blood flow increased from 0.9 +/- 0.4 l/min at rest to 5.6 +/- 1.1 l/min during exercise ($P < 0.0005$, figure 6.5.). Compared to Doppler ultrasound, use of MR cine phase-contrast techniques has the additional advantages of measuring spatial distributions of through-plane velocity over a cross section and allow concurrent quantification of flow rate, circumferentially resolved wall shear stress and lumen area.

However, given the logistic and technical aspects involved in using MRI, this method is likely to remain a research tool in exercise physiology, albeit a powerful one. Other promising

techniques related to RBF estimation include measuring partial pressure of oxygen in the urine, which reflects oxygen tension within the medulla, and near-infra red spectroscopy (Leonhardt and Landes, 1963; Petrova and Mehta, 2006).

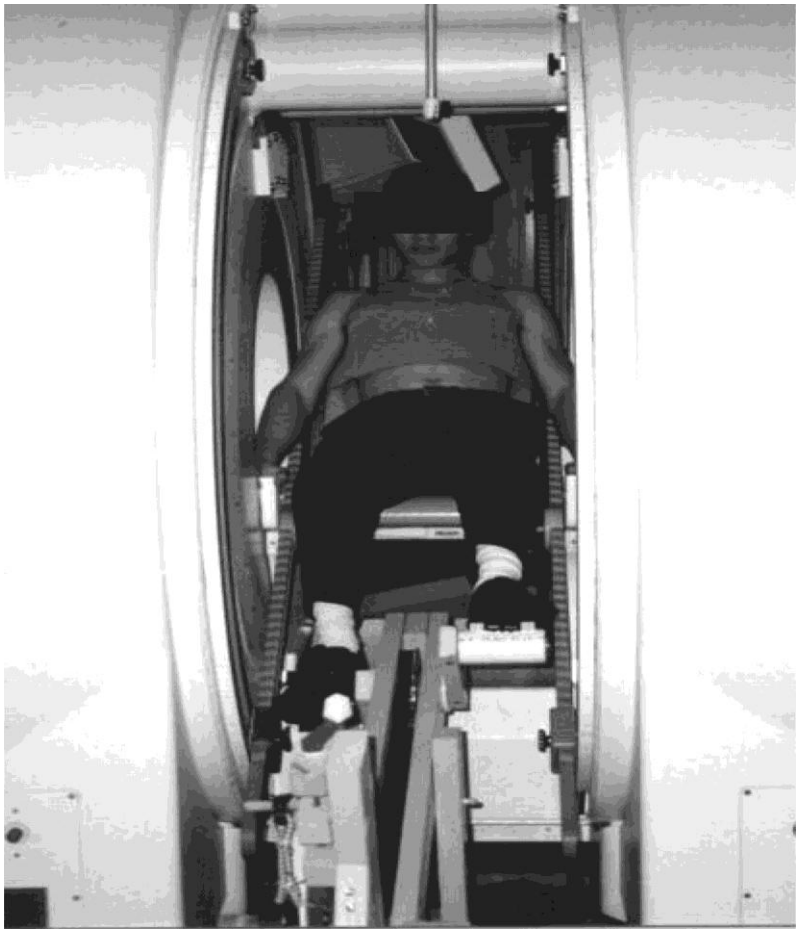

Figure 6.5. Taylor et al. used a custom MR-compatible stationary cycle in a 0.5-T open magnet MR scanner with cine phase-contrast techniques to measure blood flow velocities in the inferior vena cava of young healthy subjects at rest and during upright dynamic lower limb exercise. Subjects were strapped to an upright seat in the open magnet to allow full range of leg motion while positioning the abdomen in the centre of the magnet for optimal imaging. The cycle was then positioned and its resistance was adjusted to promote comfortable pedaling and minimize abdomen movement that could affect image capture and quality. Adopted from Taylor *et al.* 2002.

6.4.3. *A multi-factorial model of renal injury following heavy exercise.* Based upon the experimental work described in the preceding chapters, perturbations to kidney physiology during exercise cannot be regarded as merely "normal" adaptations, but should been viewed to represent physiological stress that rests on a scale of severity.

At the less severe end of the spectrum, most changes to kidney physiology in response to low intensity and/or short-duration exercise are short-lived and likely to be clinically

inconsequential. This view should continue to be respected although more work is needed to determine if repeated high-intensity short-term activity resulting in renal aberrations puts individuals at risk of future harm e.g. CKD. However, at the more severe end of the spectrum, where activity is performed at higher levels of intensity and duration, the changes to physiology can be magnified and drift into a pathological state such as AKI and its clinical sequalae (figure 6.6.). The latter scenario is likely to involve a combination of environmental and individual-specific factors that interact together within a critical time-frame – the so-called "perfect storm".

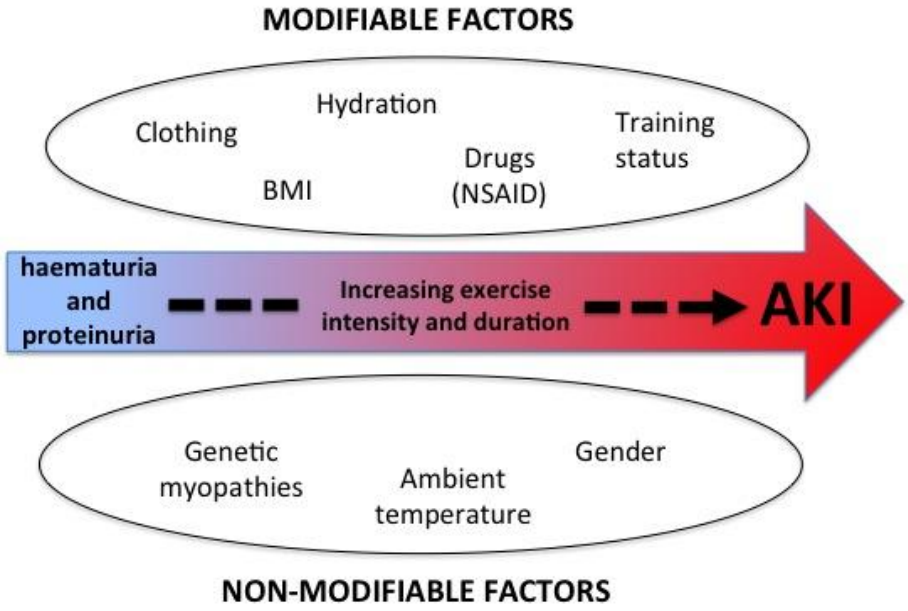

Figure 6.6. Schematic illustrating the potential mechanism of acute kidney injury through heavy physical exertion. In this setting, kidney injury is likely to exist on a scale of severity where milder activity only results in temporary phenomena such as haematuria and proteinuria. However, more strenuous activity of a higher intensity and longer duration, coupled with various adverse modifiable and non-modifiable factors increases the risk of developing significant AKI. It is at this point where "normal" physiological responses to exercise cross-over into a clinically-significant pathological state. AKI, acute kidney injury; NSAID, non-sterodial anti-inflammatory drug.

Chapters 2 and 4 have only manipulated some of these (e.g. exercise intensity, exercise-induced muscle damage and inflammation, heat-stressed environments) but other exist such as prior dehydration, clothing type, medicinal use (e.g. non-steroidal anti-inflammatory), myopathy, viral illnesses (respiratory tract infections) and body mass index (Adams *et al.*, 2012). Further experimental study is required to determine their individual and additive influences and temporal relationships. This may eventually allow for risk stratification to determine which phenotype(s) predispose to AKI following strenuous physical exertion. Such

a tool, if reliable and rapidly applied, would be useful in large populations that perform heavy exercise in adverse settings on a regular basis e.g. athletes, soldiers, fire fighters, police. An example of this can be taken from work exploring risk factors for exertional heat illness; a condition that exhibits a spectrum of severity. In a case-control study of 565 heat casualties during basic military training of Marine Corps (61 hospitalized (case subjects), 504 treated as outpatients (control subjects)), Hakre *et al.* (2004) determined risk factors for hospitalization using univariate and multivariate analyses, demographic, clinical, and laboratory factors. Of 24 potential risk factors examined, 19 were significantly associated with hospitalization. Three clinical variables (disorientation, rectal temperature, and systolic blood pressure) and three laboratory variables (serum lactate dehydrogenase, potassium and Cr concentrations) were highly predictive for hospitalization in recruits with exertional heat illness. A simple scoring system using these six variables predicted hospitalization with 87% sensitivity, 91% specificity and a likelihood ratio of 9.7 (Hakre *et al.*, 2004). In this model, it should be noted that half of the predictive variables were associated with kidney function (blood pressure, potassium and Cr), which highlights the importance of kidney health in heat illness. It is conceivable that the addition of other laboratory-measured parameters (e.g. biomarkers of kidney injury and cytokines) may improve the sensitivity and specificity of risk profiling.

6.4.4. *Wider applications of exercise testing for organ dysfunction or injury*. Although further refinement is needed, the conclusions from chapter 3 should act as a catalyst for the development of a standardised exercise test for CKD prediction. There is good justification to pursue this area, especially given that exercise testing has already been successfully applied to determine risk in the assessment of coronary artery disease; typically through the combination of a graded treadmill exercise test (e.g. Bruce protocol) with a simultaneous 12-lead electrocardiogram to detect evidence of myocardial ischaemia (Gianrossi *et al.*, 1989, Gibbons *et al.*, 2002). This develops into a powerful diagnostic and prognostic tool when the results from such investigations are combined with $\dot{V}O_{2max}$, haemodynamic responses to exercise, various forms of cardiovascular imaging and demographics. In the setting of coronary artery disease, the results of testing conclusions can be used to guide clinical decision-making in a number of situations, e.g. prediction of future cardiovascular events and the cardiovascular risk assessment for major surgical procedures. A similar scenario could be envisaged for post-exercise proteinuria positive in a CKD at-risk individual, with such information being used to dictate further investigations (e.g. renal biopsy) or secondary prevention measures (e.g. instigation of antihypertensive / antiproteinuric agents).

Apart from the heart, exercise testing for other organs has been performed to assess cardiovascular fitness in the context of other organ-specific disease states (e.g. chronic liver disease). Such information is then used to guide mortality or fitness for transplantation (Prentis *et al.*, 2012). Yet interestingly, the idea of determining the direct physiological impact of cardiovascular testing (both in the short- and long-term) upon the "fitness" of other organs (e.g. brain, liver, lungs and kidneys) and its implications has received relatively little attention. Recent evidence for cross-talk between organs during diseased states highlights the urgency to explore these potential interactions further. The cardiorenal syndrome illustrates this concept and highlights the functional interdependence between chronic heart and kidney failure to maintain homeostasis. This may occur through several putative pathways (figure 6.7.; Ronco, 2010, Darabian *et al.*, 2011).

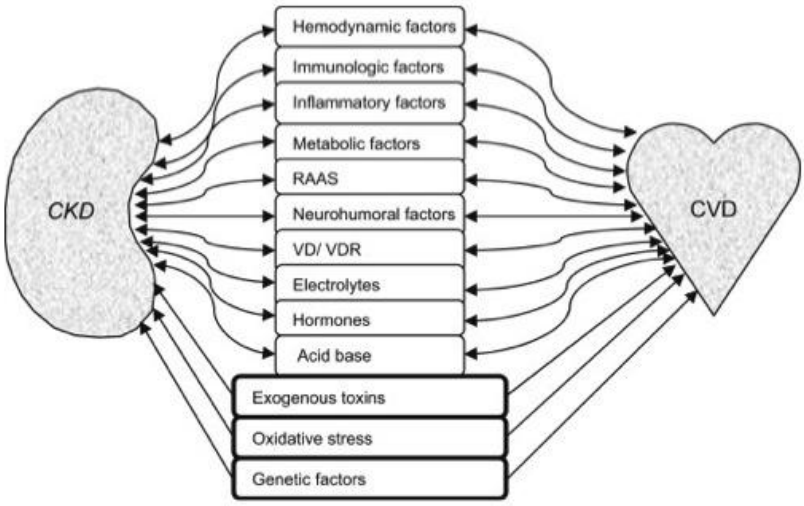

Figure 6.7. Diagram summarising the key putative pathophysiological connections in the cardiorenal syndrome. Given the recent formal recognition of this syndrome, these potential routes of communication are now being investigated in more detail. RAAS, renin–angiotensin–aldosterone system; VD/VDR, vitamin D/vitamin D receptor. Adopted from Darabian *et al*. 2011.

Haapio *et al.* (2010) have explored this complex relationship at the molecular biomarker level using plasma NGAL as a biomarker of kidney injury in patients at-risk of cardiac disease who underwent elective nuclear stress perfusion imaging. They noted that patients with detectable concentrations of plasma NGAL demonstrated greater incremental changes in natriuretic peptides, had more segmental myocardial perfusion defects, higher end-diastolic and end-systolic volumes, and higher baseline levels of the cardiac injury biomarker troponin-I following imaging (Haapio *et al.*, 2010). Although the authors stated such observations were only associations and did not extend to the directional nature of the relationships between

renal and cardiac injury, they do provide credible evidence that in a disease model cardiac and renal function are intimately linked.

Taking into account the discussion in chapter 1.2., these findings provide justification that cardio-renal relationships should be sought in healthy individuals undergoing strenuous endurance activity to elucidate the mechanisms by which physiological adaptations transgress into pathological states. McCullough *et al*. (2011) showed evidence of a cardiorenal syndrome in their marathon study that was manifested by transient right heart dilatation, elevations in blood brain natriuretic peptide and a transient decrease in renal filtration. Such investigations could also provide a platform to investigate possible molecular cross-talk between other organs during physical activity. For example, in the critically ill, AKI can co-exist with an acute lung injury and there is cross-talk which can involve a number of mechanisms including inflammatory cytokines, leukocytes, and induction of oxidative stress (Basu and Wheeler, 2013).

APPENDIX 1: EXAMPLES OF SEARCH STRATEGY TERMS USED IN CHAPTER 3

OVID MEDLINE (R) and OVID MEDLINE IN PROCESS

1. exp Exercise/ or exp Exercise Test/
2. proteinuria.mp. or exp Proteinuria/
3. albuminuria.mp. or exp Albuminuria/
4. 2 or 3
5. 1 and 4
6. chronic kidney disease.mp. or exp Renal Insufficiency, Chronic/
7. nephropathy.mp.
8. 6 or 7
9. 5 and 8
10. exercise induced albuminuria.mp.
11. exercise induced proteinuria.mp.
12. post exercise proteinuria.mp.
13. post-exercise albuminuria.mp.
14. 10 or 11 or 12 or 13

AMED

1. exp Exercise/
2. exercise testing/
3. 1 or 2
4. proteinuria.mp.
5. albuminuria.mp.
6. 4 or 5
7. 3 and 6
8. exp Kidney disease/ or chronic kidney disease.mp. or exp Kidney failure chronic/
9. nephropathy.mp.
10. 8 or 9
11. exercise induced proteinuria.mp.

12. post exercise proteinuria.mp.
13. 7 and 10
14. exercise induced albuminuria.mp.
15. post exercise albuminuria.mp.

EMBASE

1. exp exercise test/ or exp exercise/
2. proteinuria.mp. or exp proteinuria/
3. albuminuria.mp. or exp proteinuria/ or exp albuminuria/
4. 2 or 3
5. 1 and 4
6. nephropathy.mp.
7. chronic kidney disease.mp. or exp chronic kidney disease/ or exp chronic kidney failure/
8. 6 or 7
9. 5 and 8
10. post exercise albuminuria.mp.
11. post exercise proteinuria.mp.
12. exercise induced albuminuria.mp.
13. exercise induced proteinuria.mp.
14. 10 or 11 or 12 or 13

PUBMED

(((((EXERCISE OR EXERCISE TEST))) AND ((PROTEINURIA OR ALBUMINURIA)))) AND
((CHRONIC KIDNEY DISEASE OR NEPHROPATHY)) OR
(((EXERCISE INDUCED PROTEINURIA) OR EXERCISE INDUCED ALBUMINURIA) OR POST EXERCISE ALBUMINURIA) OR POST EXERCISE PROTEINURIA

COCHRANE LIBRARY

ID	SEARCH
#1	EXERCISE INDUCED PROTEINURIA
#2	EXERCISE INDUCED ALBUMINURIA
#3	POST EXERCISE PROTEINURIA
#4	POST EXERCISE ALBUMINURIA
#5	#1 OR #2 OR #3 OR #4
#6	EXERCISE OR EXERCISE TEST
#7	PROTEINURIA OR ALBUMINURIA
#8	#6 AND #7
#9	CHRONIC KIDNEY DISEASE OR NEPHROPATHY
#10	#8 AND #9

APPENDIX 2: OUTLINE OF QUADAS-2 CRITERIA AS USED IN CHAPTER 3

<table>
<tr><th>DOMAIN</th><th>PATIENT SELECTION</th><th>INDEX TEST</th><th>REFERENCE STANDARD</th><th>FLOW AND TIMING</th></tr>
<tr><td>Description</td><td>Describe methods of patient selection: Describe included patients (prior testing, presentation, intended use of index test and setting):</td><td>Describe the index test and how it was conducted and interpreted:</td><td>Describe the reference standard and how it was conducted and interpreted:</td><td>Describe any patients who did not receive the index test(s) and/or reference standard or who were excluded from the 2x2 table (refer to flow diagram): Describe the time interval and any interventions between index test(s) and reference standard:</td></tr>
<tr><td rowspan="4">Signalling questions * (yes/no/unclear)</td><td>Was a consecutive or random sample of patients enrolled?</td><td>Were the index test results interpreted without knowledge of the results of the reference standard?</td><td>Is the reference standard likely to correctly classify the target condition?</td><td>Was there an appropriate interval between index test(s) and reference standard?</td></tr>
<tr><td>Was a case-control design avoided?</td><td rowspan="3">If a threshold was used, was it pre-specified?</td><td rowspan="3">Were the reference standard results interpreted without knowledge of the results of the index test?</td><td>Did all patients receive a reference standard?</td></tr>
<tr><td>Did the study avoid inappropriate exclusions?</td><td>Did all patients receive the same reference standard?</td></tr>
<tr><td></td><td>Were all patients included in the analysis?</td></tr>
<tr><td>Risk of bias: High/low/unclear</td><td>Could the selection of patients have introduced bias?</td><td>Could the conduct or interpretation of the index test have introduced bias?</td><td>Could the reference standard, its conduct, or its interpretation have introduced bias?</td><td>Could the patient flow have introduced bias?</td></tr>
<tr><td>Concerns regarding applicability: High/low/unclear</td><td>Are there concerns that the included patients do not match the review question?</td><td>Are there concerns that the index test, its conduct, or interpretation differ from the review question?</td><td>Are there concerns that the target condition as defined by the reference standard does not match the review question?</td><td></td></tr>
</table>

* Signalling questions allow reviewer to make an assessment of bias. Extra signalling questions that are specific to the review question may be included if the reviewers believe this can assist in assessment of risk of bias.

APPENDIX 3: STUDY SPECIFIC ANCHORING STATEMENTS TO ASSIST WITH RISK OF BIAS ASSESSMENT USING QUADAS-2 IN CHAPTER 3

Domain 1: patient selection

Risk of bias: could the selection of patients have introduced bias? (high, low, unclear)

1.1 Was a consecutive or random sample of patients enrolled?

If stated explicitly, consecutive or random sampling is least likely to be associated with bias. Importantly, investigators should have no choice to determine which patients are or are not included from the population under question. A higher risk of bias is possible with non-random sampling. **Weighting: high risk of bias.**

1.2 Was a case-control design avoided?

A high degree of bias is possible in case-control studies. A hospital-based case selection may behave differently from a community-based patient selection e.g. diabetic patients who are seen in hospital clinics could be sicker or more complex. Control populations should also be healthy and be representative of a non-disease population adjusted for age and sex. Also, if authors manipulate the cohort to increase or decrease the target condition e.g. adjust anti-proteinuric agents in diabetics to limit CKD progression, this may also increase bias. In a nested case-control study where subjects are systematically selected from a defined population, there is comparatively less bias. However, the latter may narrow the spectrum of patients who undergo the index (exercise) test. **Weighting: high risk of bias.**

1.3 Did the study avoid inappropriate exclusions?

Avoiding inappropriate exclusions may result in either over- or underestimating diagnostic accuracy. Specific appropriate exclusions include: recent exercise (in term of days) prior to testing, exercising in the presence of an intercurrent illness / fever / menstruation, or exercising with chronic conditions that may decompensate and limit a complete exercise test (e.g. ischaemic heart disease, chronic obstructive pulmonary

disease). If this is not stated or not made clear, the study will be rated as having a high risk of bias. Where exclusion criteria are made explicit in the methodology, the study will be deemed of low bias. **Weighting: low risk of bias.**

1.4 Applicability: are there concerns that the included patients do not match the review question? (high, low, unclear)

The subjects tested should be derived from a population of interest as defined by the review question. Studies where there is evidence of using very select populations for the purpose of generalisations (not if they are intended to represent a defined target population) or having a low external validity, will be classified as having a low applicability.

Domain 2: index test

Risk of bias: could the conduct or interpretation of the index test have introduced bias? (high, low, unclear)

2.1 Were the index test results interpreted without knowledge of the reference standard?

Given the review question, it is expected that all studies would obtain the index test results (presence of post-exercise proteinuria) before assessing the reference standard (an objective measurement of CKD progression) at follow-up, preferably with blinding. **Weighting: low risk of bias.**

2.2 Were the index test thresholds pre-specified?

It is recognised that the cut-off or threshold for exercise proteinuria varies depending upon the type of exercise, its intensity and duration. It is also generally accepted that exercise protocols involving a high intensity and short duration yield greatest the levels of proteinuria. Studies should aim for such protocols and qualify this by explicitly defining thresholds and using exercise tests that have been previously used by independent groups. However, it is recognised that different thresholds may be used in different populations but these should be justified clearly. If this is not clear or

the authors define the cut-off based on their own study data, the study is classified at a high risk of bias. **Weighting: high risk of bias.**

2.3 Were sufficient data on application given for the test to be repeated in an independent study?

In broad terms, this should include details of a validated measure of proteinuria, methods on excluding significant proteinuria at rest, steps taken to ensure adequate pre-hydration to maximise diuresis and details of exercise protocol: type of exercise, duration, how intensity was determined and when post-exercise proteinuria was determined. If a novel exercise test was used that has not been previously described in the literature, this should be detailed in full with evidence of validation. **Weighting: high risk of bias.**

2.4 Applicability: are there concerns that the index test, its conduct, or interpretation differ from the review question? (high, low, unclear)

A poorly implemented or defined index test that also varies from the review question may have the potential to affect primary and secondary outcomes and therefore applicability. For example, if it is felt the exercise test lacked the ability to generate proteinuria, then the sensitivity or false negative rate could be affected.

<u>Domain 3: reference standard</u>

<u>Risk of bias: could the reference standard, its conduct, or its interpretation have introduced bias? (high, low, unclear)</u>

3.1 Is the reference standard likely to correctly classify the target condition?

The reference standard for the purpose of this systematic review only relates to primary outcome measures. These should be directly related to CKD progression: 1. A proteinuria level above a well-established cut-off for significant proteinuria at rest (e.g. resting proteinuria greater than 3 mg/mmol (urinary A:Cr) or 30 mg/g (urinary P:Cr) or 21 ug/min (urinary albumin excretion rate)); or 2. an increased blood creatinine concentration of 25% from baseline and / or a drop in estimated GFR

category accompanied by a 25% or greater drop in estimated GFR from baseline. These are defined by previous independent studies and international consensus groups. The reference standard should also be assessed in a period of well-being and not where illness may confound measurements. Where this has not been clarified and / or a reference standard has not been evidenced or is not familiar to the reviewer, the study will be categorised as having bias. **Weighting: high risk of bias.**

3.2 Were the reference standard results interpreted without knowledge of the results of the index test?

Knowledge of the index test results (i.e. presence or absence of post-exercise proteinuria) ***are*** required to interpret the reference standard results. However, if patients were included in the primary outcome measure who had evidence of significant proteinuria at baseline prior to exercise testing (i.e. above the reference standard), this would put the study at a high risk of bias. **Weighting: low risk of bias.**

3.3 Was sufficient information on the reference standard measurement (method on the assessment of CKD progression) given for it to be repeated in an independent study?

It is expected that authors will have used established biomarkers of CKD progression (e.g. blood creatinine and / or proteinuria) as defined by the review question. Ideally these should be referenced. Additionally, details of the assay kits used should be stated in full and again this is expected. Methods for blood creatinine determination can vary across different laboratories with methods such as the Jaffe reaction or isotope dilution-mass spectrometry. If unconventional CKD biomarkers are applied and / or in-house assay kits are used, the study will possess a high risk of bias. **Weighting: low risk of bias.**

3.4 Applicability: are there concerns that the target condition as defined by the reference standard does not match the review question? (high, low, unclear)

As stated in 3.1, the reference standard should be derived from a well-established cut-off for significant proteinuria at rest or elevations in blood creatinine / fall in estimated

GFR to indicate CKD as defined by previous independent studies or a consensus group. This should be applied consistently.

Domain 4: patient flow and timing

Risk of bias: could the patient flow have introduced bias? (high, low, unclear)

4.1 Was there an appropriate interval between the index test and reference standard?

As per review question, assessment of the primary outcome should be performed at least three months following the index test. According to international guidance, this period is sufficient to assess whether a change of a CKD biomarker represents a progression of CKD. However, significant changes assessed at a timepoint shorter than three months would not be compatible with a CKD measurement. Periods longer than three months (e.g. years) are also acceptable, providing confounding factors are accounted for or controlled for, e.g. events that could affect the reference standard such as development of disease and introduction of disease modifying medications. Both instances would place a study at a high risk of bias. Ideally, treatment of the disease under investigation should change as little as possible during the follow-up period. However, the authors recognise this may not be possible in actual practice. **Weighting: high risk of bias.**

4.2 Did all subjects receive the same reference standard?

It is expected the same reference standard (limits and assays used) should be the same at baseline and follow-up. Any deviation from this e.g. change of assay kit used, places the study at a risk of bias. **Weighting: low risk of bias.**

4.3 Were all subjects included in the final analysis?

Drop-out, subjects lost to follow up and missing data should be accounted for. There is a greater risk with studies whose follow-up period is greater than three months or extends to years. Attrition that is higher than expected (compared to other similar studies) should be treated as a high risk of bias. A cut-off of greater than 20% attrition

is defined as being high risk but this will be highly dependent on the number of subjects and length of follow-up. **Weighting: low risk of bias.**

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