EXERCISE TRAINING IN CHRONIC KIDNEY DISEASE: AN EXPLORATION OF THE EFFECTS ON SKELETAL MUSCLE MASS, FUNCTION, AND THE MOLECULAR RESPONSES

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Douglas William Gould BSc, MSc

Department of Infection, Immunity and Inflammation

University of Leicester

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Douglas W Gould

Chronic kidney disease (CKD) is a catabolic condition associated with skeletal muscle wasting and dysfunction, both of which have important clinical implications. Exercise interventions are capable of improving physical function and exercise capacity in CKD patients; with those incorporating strength training significantly improve muscle size and strength.

This thesis investigated the effects of aerobic only (AE) and combined aerobic and resistance exercise (CE) on skeletal muscle hypertrophy and function, and the molecular responses following acute unaccustomed and accustomed bouts of exercise. The primary hypothesis tested was that CE would result in greater muscle hypertrophy and increases in muscle function in comparison to aerobic AE (**Chapter 5**), and that this would occur with altered expression of genes and proteins associated with the regulation of muscle mass in CKD (**Chapters 6, and 7**).

Both AE and CE resulted in increased quadriceps muscle volume and strength, however these were substantially greater in those performing CE. Muscle biopsies from participants completing both exercise interventions demonstrated a failure to upregulate p-Akt and mRNA expression of the myogenic regulators following the initial bout of exercise indicating that CKD patients may exhibit an impaired response to the exercise stimulus in the untrained state. This response appears to be restored following 12-weeks of CE but following AE. Moreover, both groups demonstrated a substantial increase in the gene expression of inflammatory cytokines following unaccustomed exercise, however this was not observed following accustomed exercise.

This thesis demonstrates that CE produces superior increases in muscle size and strength compared to AE alone. This appears to occur through the modulation of intramuscular pathways following regular exercise and therefore highlights the importance of incorporating regular resistance exercise into exercise interventions aiming to improve muscle dysfunction in CKD.

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Responsibilities

The data presented in this thesis forms part of a larger body of work (the ExTra CKD study) and as such my responsibilities that constitute the experimental chapters of this thesis are as follows:

- Screening and recruitment of participants with the help of other members of the study team (I was involved in patient recruitment from the start of the ExTra CKD study until its completion).

- Supervise exercise sessions along with other members of the study team.

- Maintenance of the site file in accordance with NHS research procedures.

- Conduct outcome assessments, including all skeletal muscle ultrasounds in accordance with standard operating procedures and assisting with the collection and handling of skeletal muscle biopsies.

- Analysis of data including all of the imaging data (MRI and ultrasound) and laboratory analysis of the blood and skeletal muscle samples.

- Data input, statistical analysis and interpretation of all work presented in this thesis.

- Write, present and publish the work of my PhD in scientific congresses, in peer review journals, and invited presentations (listed below).

Publications and Impact

Publications

Watson EL, **Gould DW**, Xenophontos S, Clarke AL, Vogt BP, Wilkinson TJ, Viana JL & Smith AC (2017) 12-weeks combined resistance and aerobic training confers greater benefits than aerobic alone in non-dialysis CKD. *Under review in Nephrology Dialysis Transplant*

Wilkinson TJ, **Gould DW**, Watson EL & Smith AC (2017) Commentary: Renal Function Estimation and Cockcroft-Gault Formulas for Predicting Cardiovascular Mortality in Population-Based, Cardiovascular Risk, Heart Failure and Post-Myocardial Infarction Cohorts: The Heart 'OMics' in AGEing (HOMAGE) and the High-Risk Myocardial Infarction Database Initiatives. *Frontiers in Medicine (Lausanne);* 12;4:77 doi: 10.3389/fmed.2017.00077.

Gould DW, Graham-Brown MPM, Watson EL, Viana JL, & Smith, AC. (2014) Physiological benefits of exercise in pre-dialysis chronic kidney disease. *Nephrology (Carlton);* 19(9):519-27.

Conference proceedings

Oral presentations

Gould DW, Watson EL, Xenophontos S, Graham-Brown M, Wilkinson TJ, Viana JL, Smith AC. The effects of a 12-week supervised aerobic or combined exercise on muscle size, strength, and physical performance in non-dialysis CKD patients. *Renal Association UK Kidney Week 2017, Liverpool June 2017*

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Gould DW, Watson EL, Xenophontos S, Vogt BP, Wilkinson TJ, Viana JL & Smith AC. (2016) Skeletal Muscle Quality as Measured by Echo Intensity Contributes to the Loss of Muscle Strength and Functio in Patients with Non-Dilaysis CKD. *European Renal Association-European Dialysis and Transplant Association (ERA-EDTA), Vienna, May 2016.*

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Invited talks and presentations

Gould DW: Effects of exercise on muscle metabolism in pre-dialysis CKD. *International Symposium – Exercise in Chronic Kidney Disease Research and Clinical Implementation, University Institute of Maia ISMAI, Portugal, June 2017*

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Gould DW: Skeletal muscle metabolism in CKD. *UK Anaemia Clinical Study Group & Industry Meeting, Chicago US, November 2016.*

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Other impact

Gould DW & Smith AC: Motivate2Move Chapter 5 – Chronic Kidney Disease (CKD), available at https://gpcpd.walesdeanery.org/index.php/chapter-5-ckd2

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Awarded best poster in group at UK Kidney Week, Leeds, June 2015 "Association of Rectus Femoris Cross Sectional Area and Measures of Physical Function in Patients with CKD"

Table of Contents

Chapter 5 - A Rnadomised Trial on the Effects of 12-weeks Aerobic or Combined Exercise on Muscle Size and Strength in non-dialysis CKD

Chapter 6 - Skeletal Muscle Molecular Responses to Aerobic and Combined Aerobic and Resistance Exercise in non-dialysis CKD 105

List of Tables

List of Figures

List of common abbreviations

ActIIBR   Activin IIB receptor

AE   Aerobic exercise

ALMI   Appendicular lean mass index

ANOVA   Analysis of variance

BCAA   Branched chain amino acids

BIA   Bioelectrical impedance analysis

BSA   Bovine serum albumin

CI   Confidence intervals

CKD   Chronic kidney disease

CVD   Cardiovascular disease

DXA   Dual-energy x-ray absorptiometry

e1-RM   Estimated 1-repetition maximum

ESRD   End stage renal disease

FOXO   Forkhead type O

GFR   Glomerular filtration rate

IGF-1   Insulin like growth factor-1

IL-6   Interleukin-6

ISWT   Incremental shuttle walking test

KDIGO   Kidney disease improving global outcomes

MAFbx   Muscle atrophy F box

MBI   Magnitude based inferences

MCP-1   Monocyte chemoattractant protein-1

MRF   Myogenic regulatory factors

MRI   Magnetic resonance imaging

mTOR   Mechanistic target of rapamycin

mTORC1	Mechanistic target of rapamycin complex 1
MuRF1	Muscle RING finger 1
MyHC	Myosin heavy chain
P70S6K	70-kDa ribosomal protein S6 kinase
p-Akt	Phosphorylated Akt
Pax7	Paired box transcription factor 7
PEW	Protein energy wasting
PIS	Participant information sheet
QoL	Quality of life
RF-ACSA	Rectus femoris anatomical cross sectional area
RF-EI	Rectus femoris echo intensity
RNA	Ribonucleic acid
RPE	Rating of perceived exertion
RT-qPCR	Real time quantitative polymerase chain reaction
SD	Standard deviation
SMM	Skeletal muscle mass
SMMI	Skeletal muscle mass index
STS-5	Sit-to-stand 5
STS-60	Sit-to-stand 60
TTBS	Tris-buffered saline with Tween
UPS	Ubiquitin proteasome system
VO_{2peak}	Peak oxygen uptake

Chapter 1

General Introduction

1.1 Chronic Kidney Disease

Chronic kidney disease (CKD) is the term given to a group of heterogeneous disorders resulting in irreversible alterations to renal structure and function over a period of months or years (Webster, Nagler et al. 2017). CKD is defined as decreased kidney function measured by a glomerular filtration rate (GFR) <60 mL/min/1.73m^2 or markers of kidney damage, or both, present on at least two occasions for $\geq$ 3 months, irrespective of cause (Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. 2013). GFR is used to classify the disease into 5 stages: >90 mL/min/1.73m^2 (stage 1); 60-89 mL/min/1.73m^2 (stage 2); 45-59 mL/min/1.73m^2 (stage 3a); 30-44 mL/min/1.73m^2 (stage 3b); 15-29 mL/min/1.73m^2 (stage 4); and <15 mL/min/1.73m^2 (stage 5). Whilst stages 3 to 5 may be defined by estimated GFR (eGFR) alone, stages 1 and 2 also require the presence of persistent proteinuria, albuminuria, haematuria or structural abnormalities. Stage 5 (GFR <15 mL/min/1.73m^2) is referred to as end stage renal disease (ESRD), where patients require a form of renal replacement therapy (RRT) such as dialysis or transplant to maintain life (Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. 2013).

CKD is a global health problem where prevalence is estimated to be 8-16% worldwide (Jha, Garcia-Garcia et al. 2013). The UK prevalence of CKD stage 3-5 is reported to be 6% in males and 7% in women, increasing with age (Roth, Roderick et al. 2010). Whilst only a small proportion of patients with CKD will progress to ESRD the prognosis for patients is always poor and CKD is associated with increased morbidity and mortality, in addition to a number of complications including muscle wasting, anaemia, acidosis, and systemic inflammation.

Estimated GFR is an independent predictor of death, cardiovascular events, and frequency of hospitalisation (Go, Chertow et al. 2004), whilst CKD itself is associated with a wide variety of metabolic conditions including type II diabetes, cardiovascular disease (CVD) and obesity (Shlipak, Fried et al. 2005). Furthermore, patients with CKD across all stages of the disease display poor physical functioning and reduced exercise capacity (Hiraki, Yasuda et al. 2013, Painter 2005), which directly associates with all-

cause mortality (Sietsema, Amato et al. 2004, Roshanravan, Robinson-Cohen et al. 2013). The loss of physical function has numerous causes including inactivity (Roshanravan, Khatri et al. 2012), anaemia (Odden, Whooley et al. 2004), inflammation (Ortega, Rodriguez et al. 2002), and skeletal muscle dysfunction (Johansen, Shubert et al. 2003, McIntyre, Selby et al. 2006). These factors in turn, further reduce exercise capacity, culminating in a downward spiral of physical inactivity and de-conditioning associated with significantly increased cardiovascular risk (Kosmadakis, Bevington et al. 2010).

1.2 Muscle wasting and dysfunction in CKD

Skeletal muscle serves several vital functions including force production, glucose uptake, temperature regulation, energy metabolism, storage of amino acids, and more recently identified, endocrine and immune functions (Wolfe 2006). Therefore maintenance of muscle mass is of high importance, particularly in individuals suffering from chronic conditions. CKD is a catabolic state associated with metabolic derangements and skeletal muscle wasting. As such, patients with CKD commonly experience skeletal muscle wasting and dysfunction, which strongly associates with increased morbidity and mortality (Carrero, Chmielewski et al. 2008).

Whilst the loss of skeletal muscle is more prevalent in those receiving haemodialysis, evidence suggests that it starts in the early stages of the disease and is associated with increased risk of mortality, and cardiovascular events. Moreover, muscle wasting and dysfunction likely contribute to the reduced physical performance commonly reported in the patient group, which is associated with poor outcomes independent from kidney function (Roshanravan, Robinson-Cohen et al. 2013). This is important as skeletal muscle wasting and dysfunction are potentially reversible and offer a therapeutic target for intervention in a condition where in the vast majority of cases, the progressive loss of kidney function is largely irreversible. However, despite the recognised importance of skeletal muscle wasting and dysfunction in CKD populations, currently no targeted therapies to combat this complication are routinely provided.

1.3 Exercise training in CKD

Exercise training is an intervention that has been shown to beneficially impact upon both muscle mass and physical performance in CKD populations (Cheema, Chan et al. 2014, Heiwe, Jacobson 2014, Heiwe, Jacobson 2011). Indeed, in recent years exercise recommendations have started to be included in clinical practice guidelines for the treatment and management of CKD (KIDGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. 2013, K/DOQI Clinical Practice Guidelines for Cardiovascular Disease in Dialysis Patients. 2005). Despite this, the provision of exercise advice and rehabilitation programs for CKD patients in the UK is well behind that of cardiology and respiratory services (Smith, Burton 2012). This is likely due to a lack of specific guidelines on the best exercise modalities for exercise prescription, and how these should be implemented.

In non-dialysis CKD aerobic exercise has been shown to increase exercise capacity and produce cardiovascular benefits such as improvements in vascular stiffness, blood pressure control, and exert anti-inflammatory effects (Heiwe, Jacobson 2011, Heiwe, Jacobson 2014, Viana, Kosmadakis et al. 2014). However, its effects on skeletal muscle remain equivocal. Furthermore, previous work from our group suggests it may have important negative effects on skeletal muscle in CKD patients that may limit the ability to increase muscle mass (Watson, Kosmadakis et al. 2013). On the other hand, progressive resistance exercise can increase muscle size, strength and physical function in CKD populations (Cheema, Chan et al. 2014, Watson, Greening et al. 2014). Ideally, patients would perform a combination of both modes of exercise given the important benefits gained from performing both modes separately. Whilst a handful of studies have investigated the effects of combined exercise programmes incorporating a combination of aerobic and resistance exercise, reporting improvements in exercise capacity and physical performance in comparison to non-exercise control subjects (Headley, Germain et al. 2012, Greenwood, Lindup et al. 2012, Clyne, Ekholm et al. 1991), the effects of performing a combination of aerobic and resistance exercise on muscle hypertrophy has not been investigated. Moreover, no studies have investigated how the differing modes

of exercise interact with the mechanisms underpinning muscle wasting in CKD, and how this results in adaptations such as muscle hypertrophy.

1.4 Thesis structure and aims

This thesis consists of 4 experimental chapters (**Chapters 4, 5, 6, and 7**) with the overarching aim to assess the association of muscle wasting on physical performance, and investigate the effects of performing supervised aerobic (AE) or combined aerobic and resistance exercise (CE) training on skeletal muscle hypertrophy, function, and the molecular responses in patients with CKD stage 3b-5 not requiring dialysis. The primary hypothesis tested was that combined aerobic and resistance exercise would result in greater muscle hypertrophy and increases in muscle function in comparison to aerobic exercise alone, and that these adaptations would be associated with changes in the expressions of genes and proteins known to contribute to muscle wasting in CKD.

The structure of this thesis and specific aims of each chapter are as follows:

- **Chapter 2**

This literature review expands on Chapter 1, summarising the mechanisms of muscle wasting in CKD and the existing evidence for exercise increasing muscle mass and function in non-dialysis CKD, and how different exercise modes interact the mechanisms of muscle wasting.

- **Chapter 3**

This chapter describes the Exercise Training in Chronic Kidney Disease (ExTra CKD) study protocol and methods from which data appears in more than one experimental chapter.

- **Chapter 4**

This experimental chapter aimed to characterise the population of CKD participants taking part in the ExTra CKD study for the prevalence of sarcopenia, and investigates the associations between the presence of muscle wasting and imaging measures of muscle size with physical performance amongst the same cohort of non-dialysis CKD participants. It was hypothesised that the presence of muscle wasting and measures of muscle size would be independently associated with physical performance

- **Chapter 5**

Chapter 5 describes the results of the ExTra CKD study, a randomised trial that investigated the effects of performing aerobic exercise alone or in combination with progressive resistance exercise on muscle size and physical performance in participants with non-dialysis CKD stage 3b-5. It was hypothesised that CE would result in greater gains in muscle hypertrophy (measured using MRI and ultrasound) and strength than performing AE.

- **Chapter 6**

Chapter 6 investigates the molecular responses of proteins and genes involved in regulation of muscle mass following unaccustomed and accustomed exercise in CKD participants completing the ExTra CKD trial. It was hypothesised that CE would result in greater increases in phosphorylated signalling proteins that control muscle protein synthesis and reduce indicators of muscle protein degradation. Whilst AE alone would supress factors associated with protein degradation.

- **Chapter 7**

Chapter 7 aimed to investigate the gene expression of myogenic regulatory factors, myostatin, and inflammatory cytokines in the skeletal muscle of non-dialysis CKD participants in comparison to age and sex matched healthy control individuals, and how these are stimulated by performing unaccustomed and accustomed aerobic or combined

exercise. It was hypothesised that the mRNA expression of the myogenic regulators would be lower in the skeletal muscle of CKD participants, whilst the gene expression of myostatin, the activin IIB receptor and inflammatory cytokines would be upregulated. It was also hypothesised that both modes of exercise would increase the expression of the myogenic regulatory factors and IL-6 at the time of biopsy, and supress myostatin and activin IIB in CKD participants.

Chapter 2

Skeletal Muscle Wasting and Exercise in non-dialysis CKD

2.1 Skeletal muscle wasting and dysfunction in CKD

2.2 Terminology

2.2.1 Protein energy wasting

A number of similar constructs have been used to describe the catabolic state in CKD, the most prominent being, protein energy wasting (PEW), sarcopenia, and cachexia. PEW is the term proposed by the International Society of Nutrition and Metabolism to describe a syndrome consisting of nutritional and metabolic derangements that results in the loss of body protein mass and fuel stores that occurs in CKD populations (Fouque, Kalantar-Zadeh et al. 2007). PEW is the result of increased catabolic stimuli, uraemic toxins, malnutrition, and inflammation, and is commonly observed in CKD patients with an eGFR <45 mL/min/1.73m^2 and ESRD, where it is closely associated with morbidity and mortality (Obi, Qader et al. 2015, Bonanni, Mannucci et al. 2011). Importantly, muscle wasting is a hallmark of PEW, where diagnosis is based upon satisfying 3 of following criteria: abnormal serum biochemistry (low serum albumin, pre-albumin, or cholesterol), low body and/or muscle mass, and low dietary protein and/or energy intake (Obi, Qader et al. 2015).

2.2.2 Sarcopenia

Sarcopenia is the term given to the loss of skeletal muscle mass with aging, translating approximately to 'loss of flesh' (in Greek '*sarx*' meaning 'flesh' and '*penia*' meaning 'poverty') (Rosenberg 1997), and is defined as "a syndrome characterised by the progressive loss of muscle mass and strength with a risk of adverse outcomes such as physical disability, poor quality of life (QoL) and death" (Cruz-Jentoft, Baeyens et al. 2010). Sarcopenia can be identified by the loss of muscle mass, primarily assessed using dual-energy x-ray absorptiometry (DXA) or bioelectrical impedance analysis (BIA) defined by validated cut-off points alone, or in combination with functional decrements (Baumgartner, Koehler et al. 1998, Chen, Liu et al. 2014, Cruz-Jentoft, Baeyens et al. 2010, Fearon, Strasser et al. 2011).

The muscle loss that occurs in sarcopenia is likely confounded and accelerated in vulnerable populations, such as those with CKD which has been referred to as a state of premature aging (Kooman, Kotanko et al. 2014). However, given that the prevalence of CKD is highest in the elderly, distinguishing between sarcopenia and disease related muscle wasting can be difficult. Indeed, Sharma et al (2014) investigated the association of sarcopenia and CKD stage in a cohort of 11,643 National Health and Nutrition Examination Survey (NHANES) participants. The authors reported an almost linear relationship between eGFR and low muscle mass defined using DXA cut-offs, however, when adjusting for age and gender, the association disappeared and only CKD stage 4 was independently associated with sarcopenia in participants >60 years old. Nevertheless, the catabolic state and metabolic derangements associated with reduced kidney function which cause abnormal muscle protein balance (discussed below) are likely to make individuals with CKD more susceptible to the loss of muscle mass.

2.2.3 Cachexia

Cachexia is defined as "a complex metabolic syndrome associated with underlying illness and characterised by on going loss of skeletal muscle mass with or without fat mass" (Evans, Morley et al. 2008). Cachexia refers to a severe state of wasting and differs from sarcopenia as by definition it is associated with underlying illnesses such as CKD, cancer, chronic obstructive pulmonary disease, infection, and, chronic heart failure (von Haehling, Anker 2010). Indeed the diagnosis of cachexia includes the presence of a chronic disease, weight loss of 5% over the previous 3-12 months (or BMI <20 kg/m^2), and at least three of the following criteria: reduced muscle strength, fatigue, anorexia, low fat-free mass index, abnormal biochemistry, inflammation, anaemia, and low albumin (Evans, Morley et al. 2008, von Haehling, Anker 2010, Fearon, Strasser et al. 2011). All of which are commonly reported in CKD.

Clearly there is considerable overlap between the definitions of PEW, sarcopenia, and cachexia. However, currently there is no consensus regarding their utility in CKD populations or indeed other conditions associated with muscle wasting, where despite being distinct conditions, they are used interchangeably (Anker, Coats et al. 2014).

Regardless, the loss of skeletal muscle mass is central to all constructs discussed above and shares many of the same mechanisms that result in muscle wasting (discussed later on in this chapter). As a result, Anker and colleagues (2014) recently proposed the term *muscle wasting disease* to encompass muscle wasting associated with cachexia and sarcopenia of all severities associated with underlying disease. Therefore throughout this thesis the term *muscle wasting* will be used to describe the loss of muscle mass in CKD, unless specific studies are being described that use the terms outlined above.

2.3 Prevalence and clinical importance

2.3.1 Prevalence

Whilst the loss of skeletal muscle may be most prevalent and functionally severe in ESRD (McIntyre, Selby et al. 2006) evidence suggests that the wasting process starts early in the disease process. Indeed, in an earlier study, it was identified that pre-dialysis CKD patients experienced greater reductions in muscle cross sectional area compared to peritoneal- and haemodialysis patients over a one-year period (John, Sigrist et al. 2013). Furthermore, studies analysing muscle biopsies collected from the vastus lateralis muscle of non-dialysis CKD patients have shown histopathological abnormalities indicative of skeletal muscle catabolism (Heiwe, Clyne et al. 2005) and atrophy of type IIa and IIx fibres (Clyne, Esbjörnsson et al. 1993). Moreover, a recent study by Segura-Orti (2017) reported lower quadriceps muscle cross-sectional area measured using magnetic resonance imaging (MRI) in 22 patients with CKD stage 3-4 in comparison to healthy control participants.

In recent years, the prevalence of muscle wasting in non-dialysis CKD has been investigated using various criteria proposed for the identification of sarcopenia. In 11,625 participants from the Korean NHANES, Moon et al. (2015) reported that the prevalence of low muscle mass indicated by low appendicular lean mass (sum of lean mass in arms and legs) as a percentage of body mass (ALM/weight <27.24% men and <21.31% women) increased with the progression of CKD stage. The prevalence of low muscle

mass for each CKD stage was 4.3% in individuals with normal renal function or CKD stage 1, increasing to 6.3% for CKD stage 2, and 15.4% for CKD stage 3-5. In the same year, Pereira et al. (2015) reported the prevalence of sarcopenia amongst a cohort of 287 non-dialysis CKD patients, defining sarcopenia as reduced handgrip strength plus low muscle mass measured by midarm muscle circumference (<90% of a reference value), subjective global assessment, and low skeletal muscle mass index (SMMI) ((calculated as total muscle mass/height m^2) <10.76 kg/m^2 men; <6.76 kg/m^2 women) estimated by BIA. The authors reported the prevalence of sarcopenia ranging from 5.9-9.4% depending on the method used for the assessment of muscle mass, with the lowest prevalence reported with the use of BIA.

More recently, in a cohort of 100 non-dialysis CKD patients Souza and colleagues (2017) used DXA derived muscle mass indices and measures of muscle performance to investigate the prevalence of sarcopenia. When using the criteria proposed by the European Working Group on Sarcopenia in Older People (EWGSOP) (2010) of reduced muscle mass indicated by low appendicular lean mass index ((ALMI calculated as ALM/height m^2) <7.26 kg/m^2 for men and <5.5 kg/m^2 for women), reduced handgrip strength (<30kg men and <20kg women), and low gait speed over 3 meters (<0.8m/s), the authors reported the prevalence of sarcopenia of 11.9%. However, when using the criteria proposed by the Foundation for the National Institutes of Health (FNIH) Sarcopenia project (2015) where ALM is divided by BMI to identify a state of low muscle mass (<0.789 men and <0.512 women), handgrip strength (<26kg men and <16kg women), gait speed of <0.8m/s over 3 meters, a prevalence of 28.7% was reported. Importantly, the prevalence of sarcopenia using both criteria increased as CKD stage progressed with a prevalence of 34.5% in the earlier stages of CKD (stage 2 and 3a) increasing to 65.5% in CKD stages 3b-5. Moreover, when looking at low muscle mass only, the authors reported an overall prevalence of 44%. Whilst the greater prevalence of sarcopenia using the FNIH criteria suggests that BMI may affect the diagnosis of low muscle mass in patients with CKD, it is not clear what proportion of participants were diagnosed as having low muscle mass indices using this criterion versus the EWGSOP.

Finally, Zhou et al (2017) reported 44% of men and 22% of women with CKD stage 3a-5 had low muscle mass indicated by ALMI <7.26 kg/m^2 for men and <5.5 kg/m^2 for women in their cohort of 151 patients. However, when using a combination of low muscle mass and strength as proposed by the EGWSOP (2010), the overall prevalence of sarcopenia was 14%; affecting 16% of men and 8% of women. As in previous studies, the authors also reported an association between eGFR and sarcopenia prevalence (Souza, Oliveira et al. 2017, Moon, Kim et al. 2015).

2.3.2   Impact on physical performance

Muscle wasting is likely to contribute to reduced physical performance and strength, which are commonly reported in CKD populations. Again, the majority of the evidence for this comes from studies conducted in haemodialysis patients. However, there is now a growing number of studies investigating exercise capacity and physical functioning amongst patients with earlier stages of CKD, demonstrating reduced levels of physical performance in comparison to normative data and healthy controls (Padilla, Krasnoff et al. 2008) and getting progressively worse with disease progression (Hiraki, Yasuda et al. 2013, Clyne, Jogestrand et al. 1994, Faria Rde, Fernandes et al. 2013).

In the above-mentioned studies, Zhou et al (2017) reported that arm lean mass was independently associated with handgrip strength, whilst lower limb lean mass was associated with quadriceps strength in their cohort of non-dialysis CKD. The authors also reported associations between leg and trunk lean mass with functional reach and balance, respectively. Whilst, Souza et al (2017) reported that sarcopenia defined as ALM adjusted for BMI, was independently associated with low gait speed, reduced performance of activities of daily living, lower self reported physical function, and physical inactivity amongst their cohort of CKD patients stages 2-5. Moreover, the presence of muscle atrophy reported in by Segura-Orti and colleagues (2017) was independently associated with muscle strength and exercise capacity measured using 6-minute walk test.

2.3.3 Associations with mortality and co-morbidity

In addition to reduced physical performance parameters, muscle wasting is also associated with morbidity and mortality. In the above study by Pereira and colleagues (2015), sarcopenia measured as low SMMI using BIA and reduced handgrip strength independently associated with mortality, with a hazard ratio of 3.02 (95% CI 1.30 to 7.05). Moreover, muscle wasting measured as low psoas muscle index, assessed using computed-tomography was an independent predictor of major adverse cardiovascular events (hazard ratio: 3.98; 95% CI 1.65 to 9.63) amongst a cohort of 266 non-dialysis CKD participants (Harada, Suzuki et al. 2017).

Further to muscle wasting, indices of muscle performance are also associated with poor outcomes in non-dialysis CKD. In a study of 128 non-dialysis CKD patients (all stages), handgrip strength was independently associated with progression to ESRD and mortality in men and women (hazard ratio: 4.55, 95% CI 1.49 to 13.87 in men and 4.56, 95% CI 1.27 to 16.41 in women) (Chang, Wu et al. 2011). Whilst, in 385 patients with CKD stage 2-5 lower extremity physical performance measured as every 0.1 m/s decrement in gait speed was independently associated with a 26% increased risk of death, and each 1-second increase in the timed up and go (TUAG) test was also independently associated with an estimated 8% greater risk of death (Roshanravan, Robinson-Cohen et al. 2013). Interestingly, upper extremity muscle function assessed as handgrip strength was not an independent predictor of all-cause mortality.

2.4 What causes muscle wasting in CKD?

Reduced kidney function is associated with a host of metabolic derangements that are capable of stimulating muscle wasting. As such, the aetiology of the loss of muscle mass in CKD is complex and has numerous contributing factors. Metabolic acidosis is a frequently reported complication of CKD and associated with muscle wasting. Indeed, in early studies using animal models of CKD it was identified that acidosis results in muscle protein degradation and subsequent loss of muscle mass (May, Kelly et al. 1987, May,

Kelly et al. 1986, Mitch, Medina et al. 1994, Bailey, Wang et al. 1996). Interestingly, muscle protein degradation was largely attenuated by supplementing chow with sodium bicarbonate suggesting a casual relationship between acidosis and muscle protein degradation (May, Kelly et al. 1987). These findings have subsequently been confirmed in ESRD patients receiving renal replacement therapy (Graham, Reich et al. 1997, Graham, Reich et al. 1996), and more recently, non-dialysis CKD patients, where bicarbonate supplementation was shown to increase nutritional parameters and mid-arm circumference as an indicator of muscle mass (de Brito-Ashurst, Varagunam et al. 2009).

The catabolic effects of metabolic acidosis results in muscle protein degradation via the ubiquitin proteasome system (UPS) (discussed below). However, this appears to be dependent upon the presence of increased glucocorticoids (May, Kelly et al. 1986) as the presence of acidosis or elevated glucocorticoids alone were not capable of stimulating UPS activity (Price, England et al. 1994). Indeed, elevated levels of circulating glucocorticoids have been implicated in muscle wasting in a number of catabolic conditions including CKD (Wang, Mitch 2014) and result in protein degradation through impairing intracellular insulin/insulin like growth factor-1 (IGF-1) signalling, resulting in UPS activation (Price, England et al. 1994).

CKD is also associated with a state of chronic inflammation (Kato, Chmielewski et al. 2008), which has frequently been implicated in the muscle wasting observed in CKD. Indeed, cross-sectional studies have shown associations between systemic inflammation and muscle wasting in ESRD (Rymarz, Bartoszewicz et al. 2016) and non-dialysis CKD patients (Souza, Oliveira et al. 2017). The mechanisms for inflammation induced muscle wasting again appear to converge on the insulin/IGF-1 signalling pathway. Indeed, studies in animal models have shown that elevated levels of interleukin-6 (IL-6) interacts with other inflammatory cytokines and serum amyloid A to increase the expression of suppressor of cytokine signalling (SOCS3) (Zhang, Du et al. 2009) and signal regulatory protein alpha (SIRP-α) (Thomas, Dong et al. 2013), ultimately resulting in reduced signalling through the insulin/IGF-1 pathway and alterations in muscle protein metabolism. Moreover, tumour necrosis factor-alpha (TNF-α) has been shown to induce muscle wasting in CKD through the stimulation of myostatin, a potent negative regulator

of muscle mass (Zhang, Rajan et al. 2011), which exerts its effects through alterations in muscle protein metabolism.

2.5 Mechanisms of muscle wasting in CKD

2.5.1 Muscle protein turnover

Skeletal muscle mass accounts for approximately 40% of body mass and 50% of total body protein. The maintenance of muscle mass is the result of an overall balance between muscle protein synthesis and breakdown, which is in a constant dynamic state. Indeed, in a 60 kg man, total daily protein turnover is approximately 250-300g, of which 100-200g is derived from skeletal muscle (Tessari, Garibotto et al. 1996). As such, even small imbalances between protein synthesis and degradation can result in substantial muscle protein loss over time. Indeed, CKD appears to result in muscle protein loss through stimulating muscle protein degradation via the activation of caspase-3 and UPS (Du, Wang et al. 2004, Bailey, Wang et al. 1996, Rajan, Mitch 2008), whilst concurrently suppressing synthesis of new proteins. The molecular control of these processes is discussed below.

2.5.2 Protein synthesis

The suppression of protein synthesis appears to contribute to muscle wasting in CKD (Wang, Mitch 2014). Indeed, Adey et al. (2000) reported 27% lower rates in synthesis of mixed muscle proteins, and 37% lower rates for the synthesis of myosin proteins in 12 patients with CKD stage 3a-5 in comparison to healthy controls. Similarly, in an animal model of CKD, Wang and colleagues (2009) reported muscle protein synthesis rates were 12% lower in CKD animals in comparison to controls. Interestingly, CKD animals exhibited lower levels of phosphorylated mechanistic target of rapamycin (mTOR), also known as the mammalian target of rapamycin and 70-kDa ribosomal protein S6 kinase (P70S6K) in the skeletal muscle. Importantly the mTOR/P70S6K pathway is responsible

for mediating protein synthesis (described below), and therefore providing a potential mechanism for reduced protein synthesis in CKD.

2.5.2.1   Regulation of skeletal muscle protein synthesis

The process of protein synthesis involves the transcription of genetic information into messenger ribonucleic acid (mRNA), and the translation of mRNA into the protein amino acid sequence by ribosomes. This process is under strict molecular control by complex signalling pathways in response to environmental cues, such as exercise or feeding. Central to the regulation of protein synthesis is mTOR signalling (Ma, Blenis 2009, Laplante, Sabatini 2012). In mammalian cells, mTOR exists as two distinct complexes, mTOR complex 1 (mTORC1) and complex 2 (mTORC2), of which mTORC1, characterised by the presence of RAPTOR (regulatory-associated protein of mTOR) is responsible for mediating protein synthesis and cell growth (Laplante, Sabatini 2012). Early evidence for central role of mTORC1 signalling in muscle protein synthesis and muscle hypertrophy came from a mouse model of plantaris muscle overload (Bodine, Stitt et al. 2001). Chronic overload resulted in significant hypertrophy of the plantaris muscle, however treatment with the mTORC1 inhibitor rapamycin, completely abolished this effect indicating mTORC1 is indispensible for muscle cell growth.

Upstream of mTOR a number of anabolic factors including amino acids (feeding), growth factors, and exercise are capable of activating mTORC1. Insulin/IGF-1-Akt signalling (described below) indirectly activates mTORC1 through phosphorylating tuberos sclerosis 2 (TSC2) thus relieving its inhibitory effects on mTORC1 (Goldberg, Druzhevskaya et al. 2014, Pasiakos 2012) (fig 2.2). In contrast mechanical stimuli, such as exercise, directly activates mTOR signalling in an independent manner to growth factor signalling (Hamilton, Philp et al. 2010, Philp, Hamilton et al. 2011). Indeed, in response to exercise increased phosphorylation of mTOR at Ser 2448 (p-mTOR[2448]) is observed (Reynolds, Bodine et al. 2002, Dreyer, Fujita et al. 2006, Moberg, Apró et al. 2014, Zanchi, Lanacha 2008) and is therefore often used in studies of muscle metabolism as a marker of mTOR activity.

Activation of mTORC1 stimulates translation initiation through phosphorylation of a number of downstream targets (Ma, Blenis 2009). The process of translation initiation involves the assembly of the small and large ribosomal complexes with the mRNA transcript to be translated into protein. This process requires the assembly of the eukaryotic initiation factors (eIFs; eIF4E, eIF4G, and eIF4A), which are non-ribosomal proteins that help to stabilise the ribosome at the 5' cap of the mRNA (Ma, Blenis 2009). This process is dependent upon the binding of eIF4E to the 5' cap and subsequently recruiting the other eIFs, in a sequence that is regulated by 4E-BP1 (eIF4E binding protein 1). In a hypophosphorylated state, 4E-BP1 tightly binds to eIF4E, thus preventing its actions (Gingras, Raught et al. 1999). One of the ways mTORC1 mediates translation initiation is by the phosphorylation of 4E-BP1, resulting in the release of eIF4E allowing for the recruitment of eIF4G and eIF4A to form the eIF4F complex and subsequent mRNA translation (Gingras, Raught et al. 1999, Gingras, Raught et al. 2001).

Another important downstream target of mTORC1 is P70S6K, which activates downstream targets involved in regulating cell growth (Ma, Blenis 2009). Activation of P70S6K requires phosphorylation by mTORC1 at the specific Thr389 site (Hornberger, Sukhija et al. 2007). Although additional phosphorylation is required to maximally activate P70S6K (Pullen, Dennis et al. 1998, Alessi, Kozlowski et al. 1998), phosphorylation at Thr389 is most closely associated with its activity, and is therefore widely used as an indicator of mTORC1 dependent P70S6K activity (Hornberger, Sukhija et al. 2007).

Consistent with its activities as a protein kinase, P70S6K phosphorylates a number of downstream targets that are involved in regulating protein synthesis and cell growth (Ma, Blenis 2009). Most notably, P70S6K phosphorylates eIF4B resulting in its recruitment to eIF4A within the eIF4F complex, which increases its helicase activity stimulating peptide elongation (Rogers, Komar et al. 2002, Ma, Blenis 2009). Elongation involves the addition of amino acids to the growing polypeptide chain, however some mRNA species contain inhibitory secondary structures in the 5' untranslated region that prevent efficient scanning by the small ribosome subunit. Helicases are enzymes capable of unwinding the secondary mRNA structure and therefore play a crucial role in the translation process

(Ma, Blenis 2009). The primary mediator of peptide elongation is eukaryotic elongation factor 2 (eEF2), which is responsible for the translocation of the ribosome along the mRNA strand (Groppo, Richter 2009). The actions of eEF2 are inhibited when phosphorylated by eEF2 kinase, however, upon activation by mTORC1, P70S6K directly phosphorylates eEF2 kinase, therefore preventing its inhibitory actions and stimulating elongation through eEF2 (Browne, Proud 2002).

2.5.3   Protein degradation

Protein degradation is elevated in animal models and patients with CKD, often to a greater extent than the suppression in protein synthesis (May, Kelly et al. 1987, Wang, Du et al. 2009). As a result, it is thought that protein degradation is a more prominent cause of the muscle wasting that occurs in CKD (Wang, Mitch 2014). In skeletal muscle, at least four major proteolytic pathways exist including the calcium dependent calpain pathway, the lysosomal system, the UPS, and the caspase pathway. Of particular interest in CKD are the lysosomal system, UPS, and caspase-3.

2.5.3.1   The lysosomal system

The lysosomal system is primarily involved in the degradation of damaged organelles by the process of autophagy (Bonaldo, Sandri 2013). The autophagy-lysosome system is activated in muscle cells during catabolic conditions, including CKD where it is appears to be induced by myostatin (Wang, Yang et al. 2015) and contributes to mitochondrial dysfunction (Su, Klein et al. 2017). However, blocking lysosomal function in rats did not affect protein degradation rates in isolated muscles of rats with CKD (Bailey, Wang et al. 1996) suggesting that other more prominent pathways are responsible for the catabolism of skeletal muscle proteins in CKD.

2.5.3.2   The ubiquitin proteasome system (UPS)

The ATP dependent UPS plays a prominent role in muscle protein degradation in CKD (Bailey, Wang et al. 1996, Thomas, Mitch 2013). The activity of the UPS is highly

regulated by a cascade of enzymatic events in which proteins are marked for degradation via covalent attachments of chains of ubiquitin molecules. This process is initiated by activation of ubiquitin in an ATP-dependent step that is accomplished by the E1 ubiquitin-activating enzyme. The activated ubiquitin molecule can then interact with the E2 ubiquitin-carrier molecule, which subsequently interacts with the specific E3 ligase, which covalently joins the ubiquitin molecule to the target protein. This process is repeated until a chain of five ubiquitin molecules is formed that can be recognised and degraded by the 26S proteasome, which consists of a 20S catalytic core and two 19S regulatory sub units.

2.5.3.3 MuRF1 and MAFbx E3 ligases

Specificity of the UPS is provided by the E3 ligase, of which >1000 exist that recognise specific proteins or classes of proteins marking them for degradation by the proteasome (Lecker, Goldberg et al. 2006). Muscle contains two specific E3 ligases, muscle RING finger 1 (MuRF1) and muscle atrophy F box (MAFbx). Since their discovery in 2001 (Bodine, Latres et al. 2001, Gomes, Lecker et al. 2001), elevated expressions of MuRF1 and MAFbx have been identified in a number of catabolic conditions including CKD. Therefore expression of these atrogenes is frequently recognised as a marker of muscle wasting and activation of the UPS.

In skeletal muscle MuRF1 and MAFbx appear to have different cellular targets. Experiments in C2C12 myotubes and MuRF1 knockout mice have identified a primary role of MuRF1 in the degradation of myofibrillar proteins (Clarke, Drujan et al. 2007, Cohen, Brault et al. 2009). Interestingly, MuRF1 appears to target the degradation of thick myosin heavy chain proteins (MyHC), but not actin or thin filament proteins (Cohen, Brault et al. 2009). In contrast, targets of MAFbx have been identified in C2C12 cells as the myogenic regulator MyoD (Tintignac, Lagirand et al. 2005), and more recently, MyHC, and sarcomeric proteins (Lokireddy, McFarlane et al. 2011, Lokireddy, Wijesoma et al. 2012). However, to date it is unclear if these substrates are targets of the atrogenes *in vivo*.

The transcriptional regulation of the atrogenes is controlled by the phosphorylation status of forkhead type O family of transcription factors (FOXO1 and 3a). Phosphorylation of these factors by Akt renders them inactive, however, when these factors are not phosphorylated, they can translocate to the nucleus and stimulate the transcription of MuRF1 and MAFbx (Stitt, Drujan et al. 2004, Sacheck, Ohtsuka et al. 2004, Sandri, Sandri et al. 2004), and subsequently stimulate UPS muscle protein degradation.

2.5.4   Caspase-3

Whilst the UPS is responsible for degrading the bulk of intra-cellular proteins, including the myofibrillar proteins, its proteolytic actions towards these when they are present in a complex structure of muscle proteins is limited. The degradation of muscle proteins by the UPS is therefore dependent upon the cleavage of actomyosin and myofibrils into protein fragments that are rapidly degraded by the UPS (Du, Wang et al. 2004). The proteolytic actions of caspase-3 in skeletal muscle leaves a 14-kDa actin fragment that can be detected in the insoluble fraction of skeletal muscle and therefore gives an indication of muscle proteolysis (Workeneh, Rondon-Berrios et al. 2006). Indeed, evidence of caspase-3 activation has been observed in patients with burn injury and osteoarthritis where the expression of the 14-kDa fragment was strongly correlated with rate of protein degradation measured in the same muscle (Workeneh, Rondon-Berrios et al. 2006). Moreover, expression of the 14-kDa fragment has been reported in animal models and patients with CKD (Boivin, Battah et al. 2010, Workeneh, Rondon-Berrios et al. 2006, Du, Wang et al. 2004)

2.6 Insulin/IGF-1 signalling pathway

An important determinant of muscle protein metabolism is intact cellular signalling in response to anabolic stimuli. In line with this, intact insulin/IGF-1 signalling is central for the regulation of muscle protein metabolism. The binding of insulin or IGF-1 to its receptor provides a binding site for the insulin receptor substrate proteins (IRS-1 and -2). Once bound to the insulin receptor, either of these proteins can become a docking site for

Phosphoinositide 3-kinase (PI3K) (Glass 2010) leading to subsequent phosphorylation of Akt (p-Akt) which plays an important role in the control of skeletal muscle mass (Bodine, Stitt et al. 2001). Under normal conditions the binding of PI3K signals the phosphorylation of Akt, and its downstream targets mTORC1 and P70S6K, which mediate protein synthesis (described above). The phosphorylation of Akt also leads to the phosphorylation of the FOXO transcription factors leading to their exclusion from the nucleus (Glass 2010). On the other hand, decreased p-Akt can reduce protein synthesis and phosphorylation of FOXO transcription factors permitting their translocation to the nucleus, and the transcription of the ubiquitin E3 ligases MuRF1 and MAFbx (Stitt, Drujan et al. 2004, Sacheck, Ohtsuka et al. 2004, Sandri, Sandri et al. 2004) resulting in increased muscle catabolism via the UPS described above.

In CKD abnormal insulin/IGF-1 signalling pathway appears to be central to the abnormalities in muscle protein metabolism discussed above. Insulin resistance is a common feature of CKD and is frequently reported in patients with and without diabetes (Bailey 2013) where it contributes to altered protein metabolism in ESRD (Deger, Sundell et al. 2013) and non-dialysis CKD (Garibotto, Sofia et al. 2015). Bailey and colleagues (2006) investigated the role of insulin signalling in the skeletal muscle of CKD and sham rats, discovering functional abnormalities in the IRS/PI3K cascade resulting in a reduction of downstream p-Akt. Indeed, reduced levels of basal Akt phosphorylation have constituently been reported in the skeletal muscle of animal models of CKD (Wang, Du et al. 2009, Bailey, Zheng et al. 2006, Zhang, Wang et al. 2010) and patients with ESRD (Verzola, Procopio et al. 2011), which as discussed above results in reduced protein synthesis and increased protein degredation via the UPS.

2.7 Skeletal muscle amino acid metabolism

Abnormalities in skeletal muscle amino acid metabolism also appear to contribute to the altered muscle protein metabolism and wasting in CKD populations. It is well established that the branched chain amino acids (BCAA), in particular Leucine are capable of stimulating muscle protein synthesis, through direct activation of the mTORC1 signalling

pathway (Moberg, Apro et al. 2016, Rennie, Bohe et al. 2006). In early studies it was identified that haemodialysis patients exhibit depletion of free intramuscular amino acids, which is thought to be one of the initial steps in the wasting process (Bergstrom, Alvestrand et al. 1990). The depletion of intramuscular amino acids appears to be a result of metabolic acidosis, and is reversed by alkali therapy (Lofberg, Wernerman et al. 1997). Whilst depletion of intramuscular amino acids may limit protein synthesis through reduced substrate availability, the stimulation of muscle protein synthesis in response to amino acids is dependent upon the sensing of plasma essential amino acid concentrations (Bohe, Low et al. 2003).

In addition to resistance to insulin/IGF-1 signalling, animal models of CKD have also demonstrated resistance to amino acid induced anabolic signalling. Indeed, animal models of CKD and acute kidney injury exhibit suppressed mTOR signalling in response to leucine (Chen, Sood et al. 2011, McIntire, Chen et al. 2014), which appears to be worse in the presence of acidosis (McIntire, Chen et al. 2014). CKD induced acidosis appears to impair the active transport mechanisms responsible for transporting amino acids into the muscle cell. Furthermore, evidence from *in vitro* models suggests that CKD induced acidosis affects amino acid transport into the muscle cell (Evans, Nasim et al. 2007) resulting in proteolysis (Evans, Nasim et al. 2008). Therefore, both depletion of free amino acids and impaired availability are likely to contribute to abnormalities in muscle protein metabolism in CKD (Adey, Kumar et al. 2000, Nair 2005).

2.8 Myostatin signalling

Myostatin is a member of the transforming growth factor-β (TGF-β) family of secreted growth factors that is predominantly expressed in skeletal muscle. Myostatin was first identified as a potent regulator of muscle mass by McPherron and colleagues (1997) where genetic depletion of myostatin resulted in gross muscle hypertrophy. Myostatin is produced in skeletal muscle as prepromyostatin, and subsequently cleaved to produce myostatin and a propeptide (promyostatin) (Rodriguez, Vernus et al. 2014). The propeptide renders myostatin inactive by forming a latent complex, that is activated by liberating myostatin to exert its effects regulating muscle mass and function (Rodriguez,

Vernus et al. 2014). Myostatin binds to the activin receptor IIB (ActRIIB) that is present on muscle membranes with high affinity, resulting in the activation of the Type-1 activin receptor serine kinases, ALK4 or ALK5. These kinases subsequently phosphorylate Smad 2 and 3 proteins which form a heterodimeric complex with Smad4 (Han, Zhou et al. 2013, Rodriguez, Vernus et al. 2014). The activated Smad complexes then functions as key intracellular mediators for myostatin signalling as they translocate to the nucleus and initiate transcription of myostatin target genes (Derynck, Zhang et al. 1998). The phosphorylation of Smad2/3 also results in decreased p-Akt, which as discussed above affects muscle protein balance by increasing the expression of MuRF1 and MAFbx and suppressing protein synthesis through mTOR signalling (Rodriguez, Vernus et al. 2014).

Since its discovery it is known that elevated levels of myostatin result in muscle wasting and have been reported in a number of catabolic conditions including CKD. Indeed, in non-dialysis CKD, serum levels of myostatin are elevated in the early stages and increase with disease progression (Yano, Nagai et al. 2015). Moreover, elevated mRNA expression of myostatin has previously been reported in the skeletal muscle of animal models of CKD (Avin, Chen et al. 2016, Sun, Chen et al. 2006), and patients at the onset of peritoneal dialysis (Verzola, Procopio et al. 2011). Indeed, evidence from animal models of CKD has shown that myostatin is upregulated in response to inflammatory cytokines, and reduces signalling through the insulin/IGF1 pathway resulting in activation of the UPS and autophagy-lysosomal pathways. Moreover in CKD, myostatin results in the inhibition of muscle regeneration, ultimately resulting in muscle atrophy and fibrosis (Zhang, Rajan et al. 2011, Dong, Dong et al. 2017, Wang, Yang et al. 2015). Importantly, these effects appear to be prevented with the inhibition of myostatin. Indeed, Zhang et al (2011) used anti-myostatin peptibodies to suppress myostatin in animals with CKD and reported improved muscle protein metabolism (increased protein synthesis and decreased degradation), IGF-1/Akt signalling, and satellite cell function.

2.9 Muscle regeneration by satellite cells

Satellite cells are myogenic precursor cells that were first discovered by Mauro (1961) and have since been recognised for their important role in muscle repair and regeneration following insult or injury (Lepper, Partridge et al. 2011). Under resting conditions satellite cells remain quiescent in their anatomical position between the sarcolemma and basal lamina surrounding their associated muscle fibre until they are stimulated, at which point they activate, proliferate to become myoblasts and/or differentiate into new myofibres or fuse with existing ones to repair the site of injury (Snijders, Nederveen et al. 2015). In addition to their role in tissue repair, satellite cells have been proposed to contribute to muscle growth by providing new myonuclei to the existing muscle fibres in order to support the increasing cytoplasmic volume (Blaauw, Reggiani 2014, Petrella, Kim et al. 2008), however this is currently the topic of debate (Murach, White et al. 2017, McCarthy, Mula et al. 2011). The progression of satellite cells through the myogenic programme is controlled by the up- or downregulation of paired box transcription factor 7 (Pax7) and the myogenic regulatory factors (MRFs) (Seale, Sabourin et al. 2000). Pax7 is constitutively expressed in satellite cells and is often used as a marker of cell number and expansion following stimulation where it is upregulated (Seale, Sabourin et al. 2000, Snijders, Nederveen et al. 2015). Following stimulation, satellite cells begin to express Myf5, rapidly followed by MyoD indicating cell activation and proliferation. Subsequently satellite cells begin to express myogenin and downregulate Pax7, which coincides with myoblast differentiation (Snijders, Nederveen et al. 2015).

Figure 2.1 Progression of satellite cells through the myogenic programme and associated regulatory factors at each stage. (Adapted from Zammit et al. (2006)).

As skeletal muscle is a post mitotic tissue its ability to regenerate is dependent on satellite cells, therefore impairments in function can result in muscle wasting and dysfunction. Indeed, in the aforementioned study by Wang et al. (2009) the authors reported lower numbers of satellite cells surrounding muscle fibres and reduced levels of myogenic MRFs, MyoD and myogenin in comparison to control animals. Similarly, Zhang and colleagues (2010) reported reduced levels of these MRFs in skeletal muscle, and in line with the known roles of these MRFs, CKD animals also exhibit impaired satellite cell proliferation and differentiation in response to injury, resulting in myofibre atrophy and fibrosis. Moreover, when investigating the mechanisms, the authors reported that the aforementioned impairments in IGF-1/Akt signalling contribute to satellite cell dysfunction in CKD.

2.10 Summary of mechanisms

A number of metabolic derangements caused by CKD result in muscle wasting. Whilst the factors that initiate muscle wasting appear to work separately from each other, they converge on the same molecular pathways resulting in impaired insulin/IGF-1 signalling and increased rates of protein degradation via the UPS. Other mechanisms of muscle loss include myostatin signalling and impaired satellite cell function, however evidence from human studies in CKD is lacking. Figure 2.2 summarises the pathways resulting in muscle wasting in non-dialysis CKD.

Figure 2.2 Summary of the mechanisms leading to muscle wasting in non-dialysis CKD. CKD induced metabolic derangements result in suppressed insulin/IGF-1 signalling and reduced p-Akt. Low levels of p-Akt leads to reduced levels of protein synthesis through decreased mTORC1 pathway activation. Reduced levels of p-Akt also results in FOXO activation and translocation to the nucleus where it increases the transcription of the ubiquitin E3 ligases MuRF1 and MAFbx, ultimately resulting in increased protein degradation via the UPS. CKD also results in myostatin upregulation. The binding of myostatin to its ActIIB receptor on the muscle cell membrane results in the recruitment of ALK4 or ALK5, and subsequent Smad2 and 3 phosphorylation. This results in the recruitment of Smad4 to form a Smad complex that translocates into the nucleus and elicits changes in gene transcription, including the MRFs (red arrows and text indicate processes occuring in satellite cells). Myostatin binding also results in the suppression of p-Akt, resulting in suppressed protein synthesis and increased degradation.

2.11 Potential of exercise in combating muscle wasting in CKD

A number of interventions have been trialled to combat muscle wasting in CKD and other chronic conditions, including manipulation of the mechanisms involved (Zhang, Rajan et al. 2011), prevention of metabolic complications (i.e. acidosis) (de Brito-Ashurst, Varagunam et al. 2009), and pharmacological intervention (Macdonald, Marcora et al. 2007, Garibotto, Barreca et al. 1997) however to date there is no consensus regarding their efficacy at improving both muscle mass and function. One such intervention that has the potential to beneficially impact skeletal muscle at both molecular and functional levels is exercise training.

In the general population exercise has a profound effect upon skeletal muscle metabolism, and elicits a number of phenotypical adaptations that are beneficial for health. Indeed, there is now growing evidence that exercise is an intervention that has a multitude of benefits in CKD patients, including improved muscle mass, physical functioning, and exercise capacity. However, only a few studies have investigated how exercise affects the mechanisms that contribute to muscle wasting and dysfunction in CKD.

2.12 Aerobic exercise in non-dialysis CKD

Generally speaking, aerobic exercise is associated with improvements in aerobic capacity, cardiovascular function, and metabolic control. In addition to these, aerobic exercise also has the ability to impact upon skeletal muscle protein metabolism, primarily through reducing muscle protein degradation.

In non-dialysis CKD aerobic exercise has been shown to significantly increase exercise capacity measured as VO_{2peak} (Mustata, Groeneveld et al. 2011, Aoike, Baria et al. 2012, Boyce, Robergs et al. 1997, Eidemak, Haaber et al. 1997), exercise tolerance (treadmill walking time or peak workload) (Leehey, Moinuddin et al. 2009, Pechter, Ots et al. 2003,

Clyne, Ekholm et al. 1991, Kosmadakis, John et al. 2012) and anaerobic threshold (Toyama, Sugiyama et al. 2010) in addition to eliciting important cardiovascular benefits such as improvements in vascular stiffness, blood pressure control, and exerting anti-inflammatory effects (Heiwe, Jacobson 2011, Heiwe, Jacobson 2014, Viana, Kosmadakis et al. 2014). Furthermore, increases in quadriceps muscle strength have been reported following four months of aerobic exercise consisting of walking and cycling (Boyce, Robergs et al. 1997), whilst Baria et al. (2014) reported improvements in lower limb performance following 12-weeks of aerobic exercise performed thrice weekly.

While improvements in muscle function have been reported following aerobic exercise, its ability to stimulate muscle growth is equivocal. Previous work from our group investigated the effects of regular home-based walking exercise in a controlled study amongst a cohort of patients with CKD stage 4-5. Regular walking resulted in improvements in exercise capacity and elicited a number of cardioproctective benefits, including improvements in blood pressure control, cardiac function, and was anti-inflammatory; however no changes in muscle mass measured by whole body DXA were observed (Kosmadakis, John et al. 2012, Viana, Kosmadakis et al. 2014). In contrast, Baria et al (2014) reported an increase in leg lean mass (LBM) measured by DXA following 12-weeks of aerobic exercise in obese CKD patients. Methodological differences in the assessment of muscle mass (i.e. whole body versus regional measures) and modes of exercise may account for the discrepant findings between these two studies. Further work is needed to investigate the potential of aerobic exercise for improving muscle mass and function in CKD.

2.12.1.1 Effects of aerobic exercise on the mechanisms of muscle wasting in CKD

Analysis of muscle biopsies collected from participants in the study by Kosmadakis et al. (2012) offered a potential mechanistic insight into the effects of aerobic exercise on muscle growth in CKD. To investigate the effects of exercise and acidosis correction on muscle mass, participants were randomised to receive standard or additional bicarbonate. Subsequent analysis of muscle biopsies revealed that in patients receiving standard bicarbonate, walking exercise resulted in a depletion of free intramuscular amino acids,

however, this was prevented in participants receiving additional bicarbonate (Watson, Kosmadakis et al. 2013). One proposed explanation for the depletion of intramuscular amino acids in exercising patients receiving standard bicarbonate is that exercise generated hydrogen ions may result in a transient worsening of acidosis (Clapp, Bevington 2011), and therefore reduce muscle amino acid concentrations and protein metabolism through the mTORC1 pathway described above. In addition to the maintenance of amino acids concentrations exercise plus additional bicarbonate also resulted in decreased mRNA expression of MuRF1, indicating reduced catabolism via the UPS, however this occurred without improvements in IGF-1/Akt signalling (Watson, Kosmadakis et al. 2013).

A series of studies by Kopple et al investigated the effects of performing regular aerobic exercise in haemodialysis patients. In two separate exercise interventions of 10 and 18-weeks in duration, regular aerobic exercise performed 3x/week increased the gene expression of a number of IGF-1 isoforms, in addition to reducing myostatin mRNA expression, indicating changes favouring muscle anabolism (Kopple, Cohen et al. 2006, Kopple, Wang et al. 2007). Furthermore, in the cohort of participants performing 18-weeks aerobic exercise, reductions in the 14-kDa actin fragments in the insoluble fraction of muscle biopsies were observed, indicative of reduced protein catabolism via the caspase-3 system (Workeneh, Rondon-Berrios et al. 2006). However, despite these changes favouring muscle anabolism, no changes in muscle size were observed at the fibre (Kopple, Cohen et al. 2006) or whole body level (Kopple, Wang et al. 2007).

Similarly, using an animal model of CKD Wang et al (2009) investigated the effects of exercise on defects in muscle protein metabolism. Animals with CKD exhibited increased protein degradation and suppressed protein synthesis compared to control animals. The authors observed that treadmill running as a model of aerobic exercise, reduced protein degradation rates in CKD animals, which occurred with a reduction in 14-kDa actin fragment content, but not MuRF1 or MAFbx gene expression. However, whilst aerobic exercise was effective at suppressing degradation via caspase-3, it did not reverse the reduced rates of protein synthesis in CKD animals measured in isolated muscles, nor did it increase phosphorylation of the Akt/mTOR/P70S6K pathway.

2.13 Resistance exercise in CKD

In comparison to aerobic exercise, resistance training is traditionally more anabolic and has profound effects on muscle protein metabolism. In general, resistance exercise results in an increased muscle protein synthesis that remains elevated for up to 48hrs following resistance exercise, is effective at eliciting muscle hypertrophy, and improvements in muscle strength. Evidence for the effectiveness of resistance exercise in CKD populations comes from a recent systematic-review and meta-analysis that concluded resistance exercise is effective at eliciting muscle hypertrophy, and lead to improvements in strength and QoL (Cheema, Chan et al. 2014). In non-dialysis CKD patients 12-weeks of resistance exercise consisting of 3 x 20 repetitions of knee extension at 60% of the individual's 1-repetition maximum (1-RM), significantly improved muscle strength, which also corresponded to significant increases in walking capacity measured by 6-minute walk performance and functional mobility, assessed by TUAG (Heiwe, Tollback et al. 2001). However, subsequent analysis of muscle biopsies revealed no effect of the resistance exercise on histopathological variables including muscle fibre cross-sectional area (MF-CSA) and fibre type proportion within, or between exercise and control groups (Heiwe, Clyne et al. 2005).

Conversely, a series of studies by Castaneda et al (2001) reported significant increases in type I and II MF-CSA and total body potassium, with corresponding increases in strength following 12-weeks of resistance training consisting of 3 x eight repetitions at 80% of 1RM. Interestingly the increases in strength and muscle mass amongst this cohort occurred with reduced inflammatory cytokines (CRP & IL-6) and an 18% increase in IGF-1 (Castaneda, Gordon et al. 2004, Balakrishnan, Rao et al. 2010). More recently, Watson et al (2014) reported significant increases in quadriceps CSA and volume, and muscle strength following 8-weeks of lower limb progressive resistance exercise performed 3 x week at 70% of 1RM. Interestingly, the increases in quadriceps CSA and volume were associated with improvements in endurance capacity, suggesting that increases in muscle mass contribute to improvement in functional capacity. In view of the above, when performed at sufficient intensity, resistance exercise appears effective at stimulating muscle hypertrophy in non-dialysis CKD.

2.13.1 Effects of resistance exercise on the mechanisms of muscle wasting in CKD

In animal models of CKD, a series of studies have shown that muscle overloading as a model of resistance exercise increases the gene expression of IGF-1 in addition to increased phosphorylation of downstream mediators of IGF-1 signalling pathway (Sun, Chen et al. 2006, Chen, Sood et al. 2008). Similarly, the aforementioned study by Wang et al (2009) used plantaris muscle overloading in addition to treadmill running to investigate the effect of different modes of exercise on muscle protein metabolism in CKD. Similar to aerobic exercise, they reported overload was effective at reducing proteolysis. However, in contrast to aerobic exercise, overload was also effective at increasing the levels of protein synthesis in isolated muscles, which occurred with concurrent increases in intracellular signalling through the IGF-1/Akt pathway, which ultimately resulted in increased weight of the plantaris muscle indicative of increased muscle mass. Furthermore, in addition to improved muscle protein metabolism, work overload was effective at overcoming impairments in satellite cell function indicated by significant increases in the myogenic regulators, MyoD and myogenin (Wang, Du et al. 2009)

Similar findings have been reported in haemodialysis patients. Indeed, Kopple et al reported increased mRNA of a number of IGF-1 isoforms, including the IGF-1 receptor in the skeletal muscle of haemodialysis patients following 18-weeks of resistance exercise, however no change in protein degradation indicated by the 14-kDa fragment was observed. Moreover, a recent study by Molsted et al. (2015) reported increases in satellite cell number in type I fibres and myonuclear content of type II fibres of haemodialysis patients following 16-weeks of high intensity resistance exercise that resulted in muscle hypertrophy. In non-dialysis CKD, Castaneda et al (2004) reported increased serum IGF-1 following, 12-weeks of resistance exercise. Whilst low levels of serum IGF-1 have previously been reported in CKD populations, the fact that CKD results in post receptor defects in insulin/IGF-1 signalling means that the relevance of increased serum IGF-1 post exercise is unknown.

Most recently, Watson et al (2017) analysed muscle biopsies from non-dialysis CKD patients completing 8-weeks of progressive resistance exercise described above (Watson, Greening et al. 2014). Muscle biopsies were collected 24h following the first and final exercise sessions of the 8-week programme to assess the molecular responses to unaccustomed and accustomed exercise, respectively. The authors reported no change in MuRF1, MAFbx, or 14-kDa actin fragment content indicating no change in overall protein degradation. However, unexpectedly, following unaccustomed exercise, the authors reported no change in p-Akt in comparison to baseline levels, however following 8-weeks of progressive resistance exercise p-Akt was substantially upregulated. In general p-Akt is upregulated >24h following resistance exercise in healthy individuals (Mayhew, Kim et al. 2009, Constantin, Menon et al. 2013), therefore the lack of an initial response reported by Watson and colleagues may indicate impairments in IGF-1/Akt signalling in response to unaccustomed resistance exercise that appears to be overcome with regular exercise. In addition to IGF-1/Akt signalling the authors reported no changes in the MRFs, MyoD and myogenin following both unaccustomed and accustomed resistance exercise, despite reporting significant reductions in myostatin mRNA at both time points. Again, this may indicate impaired satellite cell activation, proliferation and differentiation in response to resistance exercise, which is a well-known potent stimulus of satellite cell activity in healthy individuals.

2.14 Combined aerobic and resistance exercise in CKD

Given the important benefits conferred by aerobic and resistance exercise when performed individually, combined exercise programmes have been suggested to elicit greatest benefits to patients (Heiwe, Jacobson 2011, Smart, Williams et al. 2013) however, currently few studies have evaluated the effects of combining traditional aerobic exercise programmes with progressive resistance exercise in non-dialysis CKD patients (Heiwe, Jacobson 2014). To date, a handful of studies have investigated the effects of combined exercise programmes incorporating a combination of aerobic and resistance exercise, reporting improvements in exercise capacity and physical performance in comparison to non-exercise control subjects (Headley, Germain et al. 2012, Greenwood, Lindup et al. 2012, Clyne, Ekholm et al. 1991), however, only one study has reported

improvements in thigh muscle function following a combined exercise programme (Clyne, Ekholm et al. 1991), whilst no studies have investigated the effects of combined exercise on muscle hypertrophy in non-dialysis CKD patients.

2.14.1 Effects of combined exercise on the mechanisms of muscle wasting in CKD

In line with the lack of data regarding the effects of combined exercise on muscle mass and strength in non-dialysis CKD, there is a lack of information regarding its effects on the mechanisms of muscle wasting. Gregory and colleagues (2011) reported no significant changes in the IGF-1 system in patients with CKD stage 2-4 despite noting improvements in physical performance following a 48-week intervention consisting of mixed aerobic exercise performed at 50-60% of VO_{2peak}, with the addition of resistance training that was introduced at weeks 24-48. The lack of change in circulating IGF-1 levels may be attributable to the lack of specified intensity for the resistance training component. In contrast, the aforementioned study by Kopple and colleagues reported increased IGF-1 isoforms and concurrent reductions in 14-kDa actin fragment content following 18-weeks of combined aerobic and resistance exercise in haemodialysis patients.

2.15 Summary

Chronic kidney disease is a catabolic condition associated with skeletal muscle wasting and dysfunction, which closely associates with increased mortality. Muscle wasting in CKD appears to be the result of a number of metabolic derangements caused by reduced kidney function. However, whilst the factors that initiate muscle wasting appear to work separately from each other, they converge on the same molecular pathways resulting in impaired insulin/IGF-1 signalling and protein degradation via the UPS, whilst, other mechanisms of muscle loss include myostatin signalling and impaired satellite cell function. Importantly skeletal muscle wasting and dysfunction appear to be reversible,

and therefore offer an important therapeutic target for intervention in a condition where the loss of kidney function is largely irreversible.

Exercise is an intervention that has the ability to beneficially impact skeletal muscle at both the molecular and functional levels. Overall it appears that both aerobic and resistance exercise when performed individually have important benefits and can positively impact upon the mediators of skeletal muscle atrophy in CKD. However, although aerobic exercise may reduce muscle protein catabolism, which contributes to muscle wasting in CKD populations, evidence suggests it may be insufficient to overcome the abnormalities in protein synthesis to produce hypertrophic gains. Moreover, the depletion of intramuscular free amino acids following aerobic exercise without additional bicarbonate in non-dialysis CKD patients identifies the need for further research to determine if this is a consistent response to aerobic exercise that may impair the ability to increase muscle mass when combined with other anabolic therapies (i.e. resistance exercise).

In contrast, resistance exercise appears to be effective at eliciting muscle hypertrophy in non-dialysis CKD. Although currently only few data exist investigating the interactions between resistance exercise and the mechanisms of muscle wasting in CKD, it appears that resistance exercise is effective at increasing muscle protein synthesis and overcoming the impaired IGF-1/Akt signalling exhibited by CKD populations. However, the data from Watson and colleagues (2017) indicates a possible abnormal response following unaccustomed exercise, and therefore highlights the need for regular exercise to improve IGF-1/Akt signalling in non-dialysis CKD. Currently the effects of combining aerobic and resistance exercise on muscle wasting and dysfunction are largely unknown.

Chapter 3

General methods

Part 1. ExTra CKD study protocol

Part 1 of this chapter describes the Exercise Training in Chronic Kidney Disease (ExTra CKD) study protocol and methods from which data appears in more than one experimental chapter of this thesis. The ExTraCKD study was a parallel-randomised controlled study that aimed to investigate the efficacy of performing an aerobic exercise programme alone or in combination with progressive resistance training on skeletal muscle and cardiovascular adaptations. The conception and design of the study was performed prior to the commencement of this PhD and a list of my responsibilities on the study appears earlier in this thesis. This study was registered with the ISRCTN registry (Registration Number ISRCTN36489137).

3.1 Study design

In this parallel-randomised trial, participants acted as their own control group. Non-exercise (control) data was collected during a six-week usual physical activity and treatment run-in period performed prior to randomisation. The reasons for this were two-fold; firstly, in previous studies conducted by our group, the highest drop-out rates were experienced in the control arms; and secondly, studies have consistently shown that performing regular exercise, regardless of mode provides benefits above usual care control arms in CKD, and would therefore be against clinical equipoise to include a non-exercise control arm (Huffman, Slentz et al. 2011). Following this run-in period, participants were randomised to receive either 12-weeks of supervised aerobic exercise alone (AE) or combined aerobic and resistance exercise (CE), performed 3 times a week. Outcome assessments (described below) were performed at baseline, at the end of the control period, and again at the end of the 12-week training intervention (Figure 3.1).

3.1.1 Participant recruitment and ethical approval

A total of 54 participants with CKD stages 3b-5 (eGFR <45ml/min/1.73cm^2) not requiring dialysis were recruited from Nephrology outpatient clinics at the Leicester General Hospital between December 2013 and April 2016. Eligible participants were approached in person at their routine outpatient appointment first by their consultant physician, and if they wanted more information a member of the research team gave them a copy of the participant information sheet (PIS). If interested, the individual was then contacted by telephone to answer any questions, give further information, and to arrange study visits. After having at least 48hours to consider the PIS before informed consent was taken by a member of the research team. The study received ethical approval from the National Research Ethics Committee, East Midlands-Leicester (Ref: 13/EM/0344), and University Hospitals Leicester (Ref: UHL 137056).

3.1.2 Exclusion criteria

Exclusion criteria were; significant physical impairment and/or co-morbidity (unstable hypertension or angina, potentially lethal arrhythmia, myocardial infarction in previous 6 months, active liver disease, advanced cerebral or peripheral vascular disease), poor diabetic control (HbA1c >9%), inabilities to give informed consent; BMI >40 with waist circumference >102cm for males and >88 for females (an earlier study by our group had great difficulty in obtaining usable ultrasound images and biopsies from individuals with BMI>40); and expected time to renal replacement therapy <6 months.

3.1.3 Randomisation

Following the six-week control period participants were randomly assigned to either AE or CE using the random block method, stratified for CKD stage (3b, 4 or 5). A researcher who was independent from the study protocol held the randomisation list. Recruiting researchers were blinded to randomisation schedule however, due to the nature of the intervention, patients and researchers were unable to be blinded to group allocation.

3.1.4 Sample size

The primary purpose of this study was to generate skeletal muscle biopsy and blood samples to further previous work by our group (Watson, Kosmadakis et al. 2013, Watson, Viana et al. 2017, Viana, Kosmadakis et al. 2014). As such, the study was powered on a training load to elicit a detectable physiological response following exercise. To ensure the training stimulus was sufficient to elicit a physiological response, a minimum of 21 patients (80% power, $\alpha=0.05$) performing the resistance exercise component of the study. This was based on previous work by our group (Watson, Greening et al. 2014) where an increase of 75% (600 $\pm$ 682kg) in the total weight lifted in a single training session was observed over the course of the study. To ensure groups were matched, 21 patients were also recruited into the AE group. Allowing for a 30% dropout rate, 54 patients (27 in each group) were recruited.

3.2 Exercise intervention

Randomised patients were invited to attend three supervised exercise sessions per week performed at the Leicester General Hospital for a total of 12 weeks (36 sessions in total). If participants had a planned leave of absence (i.e. holiday plans) the time missed was added onto the end to ensure they had the opportunity to attend all sessions.

3.2.1 Aerobic Exercise

The aerobic exercise component of the training programme was run as circuits that were tailored to the individual, based on ability and exercise preference. Typically sessions involved treadmill exercise, cycling, and rowing exercises. Participants were encouraged to achieve 30 minutes of exercise at a moderate intensity corresponding to 70-80% of the individuals measured maximum heart rate achieved at VO_{2peak}. Exercise intensity was monitored continuously throughout each session using a heart rate monitoring system with telemetry (Polar Team, Polar Electro (UK) Ltd.) and a rating of perceived exertion

(RPE) of 12-14 (somewhat hard). For less confident and/or unconditioned patients this target was built up to gradually over the first 1-2 weeks of the training period.

3.2.2   Combined Exercise

For patients randomised to combined exercise, the resistance exercise component was performed on 2 out of the 3 sessions and consisted of leg extension and leg press exercise performed on fixed resistance machines (Technogym, Cesen, Italy). During the assessment period patients performed a 5-Repetition Maximum (5-RM) test on each piece of equipment and prediction equations (Brzycki 1993) were used to estimate 1-Repetition Maximium (1-RM). The training load (in kilograms) was set at 70% predicted 1-RM and patients performed 3 sets of 10-12 repetitions for each exercise per session. When patients could comfortably perform 3 sets with good form the training load was increased. In an attempt to match groups as closely as possible for the total volume of exercise performed over the 12-week intervention period, on the days that resistance exercise was performed, participants performed only 20 minutes of aerobic exercise.

3.3   Outcome assessments

Participants were required to attend a number of assessment visits at baseline, post run-in period, and post exercise intervention to undergo a battery of tests (Figure 3.1). The primary outcome measure was muscle strength assessed as total weight lifted during the final exercise session and estimated one-repetition maximum (e1-RM) of the knee extensors measured at baseline, following 6-weeks usual activity and following the 12-week exercise interventions. Secondary outcomes included: magnetic resonance imaging (MRI) and ultrasound imaging of quadriceps size, muscle biopsy, body composition assessed via BIA, cardio respiratory fitness measured as peak oxygen uptake (VO_{2peak}), tests of physical function, endothelial function via flow mediated dilation, cardiac bioreactivity, fasted venous blood sample, urine sample, physical activity levels, quality of life, fatigue, and symptom perception. In accordance with the aims of this thesis (outlined in Chapter 1) the outcome measures included are described in detail below.

Figure 3.1 Flow diagram of the ExTra CKD study. Arrows indicate time points of outcome assessments listed above.

3.4 Muscle strength

Maximal knee extensor strength (kg) was measured using a 5-repetition maximum (RM) protocol on a seated leg extension machine (Technogym, Cesen, Italy) Performing a true 1-RM protocol is associated with increased risk of injury and stress on muscles and joints, which is especially true in untrained or clinical populations (Reynolds, Gordon et al. 2006, Abdul-Hameed, Rangra et al. 2012). In CKD the prevalence of bone disease puts them at risk of fracture (Kim, Long et al. 2016) and spontaneous quadriceps tendon ruptures have previously been reported (Shah 2002). Therefore, the use of a 5-RM protocol allowing for the estimation of 1-RM is suggested in this population (Johansen 2005). Estimated 1-RM (e1-RM) was used to assess participants muscle strength at baseline and in response to AE or CE. For participants performing CE, e1-RM was used to set and alter training load as necessary.

Participants performed a warm up of 5 repetitions at ~50%, and then ~70% of their predicted 5-RM through a full range of movement. Following each successful completion of 5 repetitions, patients rested for 3 minutes and the weight was increased by 2.5kg or 5kg depending on the difficulty of the previous attempt. The test was complete when the individual could not perform 5 repetitions through the full range of motion. Estimated 1-RM was then calculated using the equation by Brzycki (1993), which has been shown to accurately predict maximal strength in older adults and clinical populations.

Total weight lifted by those performing CE during the exercise sessions was calculated as weight x repetitions x sets.

3.5 Magnetic Resonance Imaging (MRI) measurement of quadriceps muscle volume

MRI is often considered the gold standard for the assessment of muscle size, and was used to assess the association with quadriceps muscle volume and physical performance (Chapter 4) and changes in muscle volume in response to AE or CE (Chapter 5).

3.5.1 Image Acquisition

Axial plane scans of participant's quadriceps muscles were performed, from the proximal border of the patella to the superior aspect of the femur using a 3 Tesla HD MRI scanner (Siemens Skyra). A T1-weighted turbo spin-echo sequence was used with the following parameters: slice thickness = 5mm with no gap between slices; repetition time/echo time = 873ms/14ms; field of view = 450x309.4mm; in-plane resolution = 0.879x0.879mm. Participants lay supine on the MRI bed and the radiofrequency coil was placed over their thighs and secured by a radiographer. Participants were then positioned in the scanner so that the isocentre of the magnetic field was located at mid-thigh and requested to lie still for the duration of the scan (~10 mins). Images were obtained with two sequential acquisitions of the upper and lower thigh to ensure full coverage of the quadriceps muscle group.

3.5.2 Measurement of quadriceps volume

Quadriceps muscle volume was measured on 10mm thick slices of the right leg of each participant. Slices comprising rectus femoris, vastus lateralis, vastus medialis, and vastus intermedius were manually outlined around the facial boundary (Figure 3.3), from the first distal slice where RF was visible and every slice thereafter to the most proximal slice containing VM using online image analysis software (Jim, Xinapse Systems Ltd, UK). Where possible individual muscles were outlined, and visible fat or connective tissue within the measurement region avoided. Muscle volume data was then automatically calculated by the software using the cross sectional area from all measured slices and the known slice thickness. Images were carefully inspected to exclude any overlapping slices that may have occurred around the midpoint of the scan due to the two separate

acquisitions. All scans were anonymised by a researcher independent from the study protocols and were unblinded once all scans had been analysed.

Figure 3.2 Representative magnetic resonance image of the quadriceps muscle group. *Rectus femoris (RF), vastus lateralis (VL), vastus intermedius (VI)* and *vastus medialis (VM)* highlighted.

3.6 Ultrasound measures of muscle architecture

Skeletal muscle ultrasound was used to assess rectus femoris muscle size and composition. Data of which is presented in Chapters 4 and 5 that assess the associations between ultrasound measures and physical performance, and investigate the effects of AE or CE on muscle hypertrophy, respectively.

3.6.1 Rectus femoris anatomical cross sectional area and architecture

Rectus femoris anatomical CSA (RF-ACSA) was measured from participants right leg using B-mode 2D ultrasonography (Hitachi EUB-6500; probe frequency, 7.5 MHz) under resting conditions with the participant lying prone at a 45° angle. Imaging was performed at the midpoint between the greater trochanter and superior aspect of the patella, which was measured prior to conducting the imaging. The point of the scan marked with pen and recorded for subsequent measurements. Ample contact gel was applied to the area

and ultrasound transducer. The transducer was placed on the skin, perpendicular to the long axis of the thigh, with minimal pressure to avoid exerting compression on the muscle. Participants were asked to gently contract and relax their quadriceps to delineate parameters of the rectus femoris prior to image acquisition. RF-ACSA was then calculated by outlining echogenic facial line of the rectus femoris using the track ball cursor on a frozen image (Figure 3.2). RF-CSA was calculated as an average of three consecutive measurements with <10% variation.

3.6.2 Echo intensity

Echo intensity allows for the quantification of muscle quality based on greyscale analysis using imaging software. EI estimates the quantity of intramuscular adipose and connective tissue based on the brightness scale of the ultrasound image. Lean tissue has low echogenicity, whilst intramuscular fat and connective tissue within the muscle has high echogenicity. Saved images from RF-ACSA measures were used to determine rectus femoris EI using Image J software. Following delineation of the RF, the pixel count was determined giving a score based on arbitrary units ranging from 0 to 256 (0 = black; 256 = white). Skeletal muscle of 'better quality' with more lean tissue and less fat or connective tissue appears darker and therefore has a lower echo intensity (Young, Jenkins et al. 2015).

Figure 3.3 Representative image of rectus femoris CSA measured by 2D B-mode ultrasound.

3.7 BIA measures of skeletal muscle mass

BIA offers a quick and simple measure of muscle mass, and cut-off values for skeletal muscle mass indices are included in consensus statements for the diagnosis of sarcopenia and cachexia (Chen, Liu et al. 2014, Cruz-Jentoft, Baeyens et al. 2010, Fearon, Strasser et al. 2011). BIA derived cut-off values were used to identify participants with low skeletal muscle mass (indicative of muscle wasting) in Chapter 4 and assess the independent associations between sarcopenia and physical performance.

Whole body and segmental (right arm, left arm, trunk, right leg and left leg) lean and muscle mass were measured using 3-compartmental (3-C) Direct Segmental Multi-frequency BIA (InBody370, InBody Bldg, Korea). The 3-C model (fat mass, lean mass, and water) incorporates total body water into the assessment, and therefore controls for interindividual variation in hydration status. This makes it a more accurate method for body composition assessment in CKD patients who are susceptible to fluid imbalances (Carrero, Johansen et al. 2016).

The test lasted ~30 seconds and was carried out in accordance with the manufacturers' instructions. Briefly, participants were stood upright on the analyser's foot plates with bare feet, with legs apart and arms apart gripping the held hand electrodes in each hand. The device then performed 15 impedance measurements using 3 different frequencies (5 kHz, 50 kHz and 250 kHz) at each of the 5 segments to estimate total and segmental fat mass, soft lean mass, fat free mass, and skeletal muscle mass. All body composition assessments were performed following an overnight fast.

3.8 Cardiorespiratory fitness measured as peak oxygen uptake (VO$_{2peak}$)

3.8.1 Ramp test protocol

Peak oxygen uptake (VO$_{2peak}$) represents the integrative capacity of the respiratory system, cardiovascular system and skeletal muscles to uptake, transport and use O$_2$, respectively. Therefore direct measurement of VO$_{2peak}$ is considered the gold standard for the assessment of cardiorespiratory fitness. Peak cardiorespiratory fitness was assessed using symptom limited, graded, maximal cardiopulmonary exercise tests performed on a cycle ergometer (Lode Excalibur Sport, Gronigen, the Netherlands).

Participants were instructed to cycle for as long as possible at or above 60 revolutions per minute (RPM). Following a 3 minute warm up of steady cycling at 30 W, work-rate was increased by 1 W every 4 seconds (15 W/minute) using a ramp protocol. Throughout the test, electrocardiograph (ECG) and blood pressure were continuously monitored by an experienced cardiac nurse or doctor. The test was stopped when the participant reached volitional exhaustion; was unable to maintain a RPM ≥ 60 despite vigorous encouragement, or at the discretion of the medical professional. If the test was stopped for medical reasons, patients who had no previous reports of significant arrhythmias, ischemia or symptoms were referred for further investigations, and if necessary excluded from taking further part in the study.

3.8.2 Measurement of VO$_{2peak}$

Oxygen consumption (VO$_2$) was measured by breath-by-breath sampling (Cortex Metalyzer, Leipzig, Germay) measured continuously throughout the exercise tests. Prior to conducting each assessment, the gas analyser was calibrated using known concentrations of gases (17.10% O$_2$ and 5% CO$_2$) and the volume transducer using a 3 L Hans Rudolph syringe. VO$_{2peak}$ was subsequently calculated as the highest 20-second value achieved during the test.

3.9 Incremental shuttle walking test

The incremental shuttle walking test (ISWT) (Singh, Morgan et al. 1992) was used to assess exercise capacity and walking performance. This test has been used extensively to assess exercise capacity in chronic obstructive pulmonary disease (COPD) and other chronic disease populations including CKD (Watson, Greening et al. 2014, Greenwood, Lindup et al. 2012).

The ISWT is externally paced and requires the participant walk up and down a 10 meter course, which is identified by two cones placed 9 meters apart, allowing 0.5m at either end for turning. The speed at which the participant walked was controlled by an audible beep played on a CD. The ISWT is maximally progressive starting at 0.5 meters per second (m/s) and increasing by 0.17 m/s each minute for 12 minutes, up to a final speed of 2.37 m/s (5.3mph). Standardised instructions were played from the CD at the start of each test instructing the participant to walk along the course, turning around the cones at either end in time with the audio signals from the CD player. If participants walking speed slowed behind that of the CD, they were verbally instructed to catch up, but no further encouragement was given. If the participant slowed behind the CD for two consecutive shuttles the test was terminated. The test was terminated if the participant failed to keep up with the pace as described above, or reached volitional exhaustion. The distance walked in meters during the test was then taken as a measure of exercise capacity.

3.10 Sit-to-stand tests

Physical function was determined using the sit-to-stand 5 (STS-5), a surrogate measure or muscular power and the sit-to-stand 60 (STS-60), a surrogate measure of muscular endurance. Moving from a seated position to standing requires lower extremity strength, and can therefore be used as an indicator of lower-extremity physical function in this population (Koufaki, Mercer 2009, Painter, Marcus 2013). Participants start from a seated position on a chair (height 17 inches [43.2cm]) with feet flat and knees bent at 90°. With

their hands across their chest, the participant stands up fully and returns to the seated position, corresponding to one repetition. For the STS-5, participants were asked to perform five repetitions as fast as possible, and the time taken was recorded when the participant sat back down on the fifth repetition. For the STS-60, participants were asked to perform as many repetitions as possible in 60 seconds. The number of full repetitions performed was recorded.

3.11 Skeletal muscle biopsy

Muscle biopsies were performed by a trained physician at the Leicester General Hospital and were obtained from consenting patients on 3 occasions under fasted conditions (see Figure 3.1). A resting sample was obtained prior to the commencement of the patients first exercise session of the intervention providing baseline information of the patients status against which the other biopsies are compared (termed baseline). A second biopsy was obtained 24 hours (h) after the first training session to provide information regarding the acute effects of unaccustomed exercise in an untrained state (termed untrained). The third biopsy was obtained 24h following the final exercise session of the intervention to investigate the responses to accustomed exercise in the trained state (termed trained). The biopsy sampling timepoints at 24h post exercise have been used in previous work from our group (Watson, Viana et al. 2017) and were chosen to allow for comparisons between studies in addition to patient acceptability. Whilst a number of signalling events are likely to have returned to baseline or been missed with a single timepoint at 24h, muscle protein synthesis is elevated for up to 48h following exercise (Phillips, Tipton et al. 2007) with studies showing that the key signalling events regulating muscle protein synthesis are upregulated 24 hours following acute exercise (Mayhew, Kim et al. 2009, Mayhew, Hornberger et al. 2009, Constantin, Menon et al. 2013).

3.11.1 Vastus Lateralis needle biopsy procedure

Biopsies were taken from the middle portion of the vastus lateralis muscle of the individuals' right leg using a 12-gauge microbiopsy needle. With the patient in a supine

position the site at which the biopsy would be obtained was identified, the area was cleaned using iodine solution. After cleaning, the area was anesthetised with 5ml of 2% lignocaine, which was administered superficially under the skin and then the fascia. A small incision (<0.5cm) was made through the skin and subcutaneous tissue, and the needle inserted to the fascia and a small sample (~80mg) of muscle collected. The sample was immediately extracted from the needle, and dissected of any visible fat or connective tissue and frozen in liquid nitrogen. The frozen tissue was then placed in a cryotube and placed back into liquid nitrogen. This was performed for up to a maximum of three passages using the same incision to ensure enough tissue was obtained. Pressure was applied following each passage and following the final passage steri-strips were placed over the incision and a self-adhesive bandage applied to prevent bruising. For each subsequent biopsy, a new incision was made at least 2cm above or below the initial site. In the first 6 patients who consented to the muscle biopsy procedure, one small piece of fresh tissue (~30mg) was mounted on cork (~1.5cm^2) using Tissue-Tek O.C.T and frozen in isopentane that had been pre-cooled in liquid nitrogen with the intention of using these samples for immunohistochemical analyses. All samples were stored in liquid nitrogen until processing described below.

3.12 Blood sampling

Fasted blood samples were obtained from participants on a total of six occasions over the course of the study. A rested sample was collected from participants at one assessment visit at each of the three assessment time points. An additional three samples were obtained from consenting participants prior to each of the muscle biopsies. On each occasion, ~30ml of venous blood was taken from a superficial antecubital vein and collected in S-Monovettes® (Sarstedt Ltd., Leicester, UK) containing K2 EDTA-Gel anticoagulant or Trisodium citrate. Samples collected in EDTA were centrifuged at 2500g for 15 minutes at 4°C, whilst samples collected in Trisodium citrate were centrifuged twice at 2500g for 15 minutes at room temperature. The resulting plasma was removed into 1ml aliquots and stored at -80°C until analysis.

Part 2. Laboratory analyses

Part 2 of this chapter describes the laboratory methods that appear in more than one experimental chapter. The statistical analysis used to investigate the effects of exercise on intramuscular pathways is consistent across multiple chapters and is therefore described in this section. For convenience, microcentrifuge speeds are reported in rpm.

3.13 Tissue processing

3.13.1 Frozen muscle tissue

Muscle tissue stored in liquid nitrogen was divided for the separate analyses using a Cellcrusher™ (Stratech Scientific Limited, Suffolk, UK) pre cooled in liquid nitrogen. The Cellcrusher™ was removed from liquid nitrogen into a tray of dry ice, the frozen sample was then rapidly placed into the device and pulverised by striking the device with a mallet. The sample was then retrieved using a stainless steel spoon pre chilled in liquid nitrogen and the appropriate amount used for the analyses described below. The remainder of the tissue was then immediately returned into liquid nitrogen for storage. Samples frozen in isopentane were cut by trained biomedical scientists using a cryostat at -20°C at the Histology Facility in the Clinical Sciences department at the Leicester Royal Infirmary and used to extract RNA as described below.

3.14 RNA techniques and analysis

3.14.1 RNA extraction

RNA was extracted from the muscle biopsies of CKD patients using TRIzol® based on the method first described by Chomczynski and Sacchi (1987). Approximatley 10mg of tissue was homogenised (MP FastPrep-24® 5G, MP Biomedicals) in 1.5ml of Trizol and

incubated for 15 minutes at room temperature. Following incubation, tubes were centrifuged at 13000rpm for 15 minutes at 4°C to remove any insoluble material and the Trizol was transferred to a new sterile tube. Chloroform (200μl/ml) was added and tubes vortexed thoroughly until the substances mixed, before a 10 minute incubation at room temperature. Tubes were then centrifuged at 13,000rpm for 15 minutes at 4°C resulting in the formation of three separate phases, of which ~80% of the aqueous phase containing RNA was removed to a separate tube. Following isolation of the aqueous phase, 500μl/ml of Isopropanol was added to precipitate the RNA. Tubes were vortexed and incubated at room temperature for 10 minutes before being centrifuged at 10,000rpm for 10 minutes at 4°C to pellet the RNA. The resulting pellet was washed in 200μl of 75% ethanol and centrifuged again at 10,000rpm for 10 minutes at 4°C. The ethanol was then discarded and the RNA pellet was left to air dry for 10-15minutes. The pellet was then dissolved in 20μl autoclaved DI water, and the RNA concentration was quantified in 1μl of RNA using a NanoPhotometer® spectrophotometer (Implen GmbH, Munich, Germany) before being stored at -80°C until analysis.

3.14.2 Reverse transcription

Reverse transcription of RNA to complementary DNA (cDNA) was performed on 1μg of RNA in a 20μl reaction using the Reverse Transcription System (Promega, Hampshire, UK). RNA was heated at 70°C for 10 minutes in order to remove any secondary structure before being centrifuged briefly and placed on ice. A single 20μl reaction was prepared as shown in Table 3.1. Tubes were then heated at 42°C for 1 hour, 95°C for 5 minutes and 4°C for 5 minutes in a controlled heat block. The resulting cDNA was stored at -20°C until analysis. Additional reactions were performed where AMV was omitted to in order to determine if there was any genomic DNA contamination.

Table 3.1. Reverse transcription reaction

Reagent	Volume (µl)
MgCl$_2$	4
Reverse Transcription 10X Buffer	2
dNTP Mixture	2
Recombinant RNasin® Ribonuclease Inhibitor	0.5
AMV Reverse Transcriptase	15 Units
Primers*	1
RNA	1µg
Nuclease-Free Water	to 20µl final volume

*A 1:1 combination of random and oligo dT primers were used in the reaction buffer.

3.14.3 Identification of appropriate housekeeping genes

In order to control for technical variation when performing PCR, it is important to normalise the mRNA expression of the genes of interest to a housekeeping gene of which the expression remains stable over the experimental conditions. Housekeeping genes commonly used to normalise gene expression in skeletal muscle following acute exercise include, β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-microglobulin, cyclophilin (CYC) and 18S ribosomal RNA. However, endurance and resistance exercise may have differential effects on the expression of these commonly used reference genes, potentially making them unsuitable for the comparison between differing modes. In order to identify the most appropriate housekeeping genes that remain stable across both exercise modes, human endogenous control plates (TaqMan® Arrray – Well FAST plate, Applied Biosystems, Warrington, UK, 4426696) were used to screen 32 different reference genes across both exercise interventions. Using the cDNA from one patient in each exercise intervention the most appropriate reference genes were identified based on having a high expression and low co-efficient of variance (Table 3.2). Based on these parameters, primers and probes were purchased for 18S ribosomal RNA (18S), beta-2-microglobulin (B2M), actin beta (ACTB), cancer susceptibility candidate 3 (CASC30), and POP4 and the stability of the expression of these genes were tested in a further 3 patients from each experimental condition. From these experiments 18S was

selected as the most suitable reference gene as it was constitutively expressed and showed the lowest variance between both experimental conditions

Table 3.2 Results of endogenous control plate showing the co-efficient of variation (CV) for housekeeping genes over aerobic and combined exercise conditions

Gene	Aerobic CV (%)	Combined CV (%)	Gene	Aerobic CV (%)	Combined CV (%)
18S	3.43	2.18	CASC3	1.88	2.9
GAPDH	4.75	6.42	CDKN1A	3.42	4.63
HPRT1	1.79	3.18	CDKN1B	1.91	3.19
GUSB	2.97	3.95	GADD45A	6.61	4.0
ACTB	0.3	2.93	PUM1	1.1	3.32
B2M	1.49	3.88	PSMC4	2.04	3.6
HMBS	2.65	4.47	EIF2B1	1.25	3.72
IPO8	1.06	3.07	PES1	2.29	3.37
PGK1	3.55	4.55	ABL1	0.81	3.41
RPLPO	1.37	3.98	ELF1	1.02	3.58
TBP	2.2	4.02	MT-ATP6	6.19	7.11
TFRC	0.39	3.29	MRPL19	2.67	4.2
UBC	0.36	3.83	POP4	1.39	3.1
YWHAZ	1.99	2.96	RPL37A	5.12	4.69
PPIA	3.67	4.01	RPL30	3.02	4.11
POLR2A	1.87	3.26	RPS17	2.34	3.61

3.14.4 Real-time qPCR

Real-time qPCR was performed using an Applied Biosystems Fast 7500 Real-Time PCR system and software package, and pre-designed gene specific amplification primers and probes (TaqMan Gene Expression Assays, Applied Biosystems, Warrington, UK). PCR reactions were performed in 20μl volumes consisting of 10μl TaqMan Gene Expression Master Mix (Applied Biosystems, Warrington, UK,), 1μl TaqMan Gene Expression Assay and 8μl nuclease-free water. 1μl of cDNA or water (if a negative control) was

added once 19µl of the master mix detailed above had been applied to the plate. The PCR profile for all genes consisted of one cycle at 50°C for 2 minutes, followed by a denaturing cycle at 95°C for 10 minutes and 40 cycles of denaturing at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute.

Samples were analysed in duplicate and 'blank' reactions used to identify any contamination. All samples from the same patient were analysed on the same plate for both the target and housekeeping genes to allow for direct comparisons. A control sample was included on every plate for each gene of interest and CV calculated to determine the variance between each experimental plate for the specific gene of interest reported in Chapters 6 and 7 (Table 3.3). The Ct values from the genes of interest were normalized to the Ct values of 18S as the reference gene, and relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method which sets the baseline value to 1.0 (Pfaffl 2001).

Table 3.3 Varience between experimental plates for RT-PCR experiments reported in Chapters 6 and 7

Gene of Interest	CV (%) Between Experimental Plates
MuRF1	1.0%
MAFbx	2.3%
Myostatin	3.9%
ActIIBR	3.1%
Pax7	3.2%
MyoD	2.8%
Myf5	3.2%
Myogenin	3.2%
IL-6	4.2%
TNF-α	2.3%
MCP-1	2.5%

3.15 Protein techniques

3.15.1 Sample preparation for western blot analysis

Approximately 15-20mg/ww muscle tissue was homogenized using the MP FastPrep-24$^\circledR$ 5G (MP Biomedicals, Lysing matrix D) in 18μl/mg of ice-cold lysis buffer (137mM NaCl$_2$, 2.7mM KCl, 1mM MgCl$_2$, 50mM Tris (pH 7.5), 1mM EDTA (pH 8), 1mM EGTA, 0.1% w/v β-Mercaptoethanol, 10% w/v Glycerol, 10% v/v Triton X-100, 1μg/ml Pepstatin A, 0.2mM PMSF, 1μg/ml Leupeptin, 1mM Benzamidine, 1% v/v phosphatase inhibitor-3 (Sigma Aldrich, UK) and ultrapure water to 10ml), then rotated end over end for 90 minutes at 4°C, and microcentrifuged at 13,000rpm for 15 minutes at 4°C. The resulting supernatant was removed to a new tube and stored at -80°C in 50μl aliquots for later analysis. The remaining pellet was retained and stored at -80°C for later determination of the 14-kDa actin fragment concentration.

3.15.2 Bio-Rad Detergent Compatible (DC) Assay

The protein concentration of the muscle lysates was determined using the Bio-Rad DC Protein Assay Kit (BioRad, UK). Immediately prior to use, 20μl of Reagent S was mixed with 1ml of Reagent A to produce Reagent AS. The lysates were diluted 1:1 v/v with 1% IGEPAL CA-360 detergent (Sigma, Dorset, UK) to bring them into the range of the standard curve. Standards (0-20μg/ml) and samples were pipetted in 5μl volumes in triplicate into a 96-well plate. 25μl of Reagent AS was then added to each well, followed by 200μl of Reagent B. The plate was incubated for 15 minutes at room temperature and read at 750nm on a Multiskan$^{™}$ FC spectrophotometer (Thermo Scientific, UK).

3.15.3 Folin Lowry Assay

The protein concentration of the pellets retained from lysate preparation was determined using the Folin Lowry assay. Pellets were dissolved in 1ml of 0.5M sodium hydroxide at 70°C in a water bath for 30 minutes. Immediately before use, Folin Reagents A and B were mixed 50:1 to give Reagent C, and Folin Ciocalteu's phenol reagent (Sigma, Dorset,

UK, 47641) was diluted 1:2 with water. Muscle biopsy samples were diluted 1:10 using 0.5M sodium hydroxide to bring them into the standard calibration range (0-500µg/ml). 50µl of standards and samples were then mixed with 600µl of Reagent C and immediately vortexed. After a 10 minute incubation, 60µl of the diluted Folin Ciocalteu was added with immediate vortexing, and tubes were incubated for a further 40 minutes at room temperature before being read at 660nm on a Multiskan™ FC spectrophotometer (Thermo Scientific, UK).

3.15.4 General western blot procedure

The appropriate volume of protein was mixed in an equal volume with Laemmli reducing sample buffer (see Appendix A) and heated at 100°C for 5 minutes. Samples were briefly microcentrifuged and 30-50µg of protein loaded on acrylamide gels (Appendix A) for size dependent separation. A molecular weight marker (Full-Range Molecular Markers, GE Health Care Life Sciences, Little Chalfont, UK) was also loaded into one of the wells for confirmation of size of the protein of interest. Complete samples from each patient were loaded onto the same gel and run alongside patient samples from the other experimental group.

Electrophoresis was performed at 200 V for 40-60 minutes depending on the size of the protein of interest. Once the gels had been run, they were placed into transfer buffer along with blotting paper and nitrocellulose membrane and then transferred at 100 V for 60 minutes or 80 V for 120 minutes (Phospho ^{Ser2448} mTORC1). Following transfer, membranes were blocked for 1 hour at room temperature in 5% non-fat dry milk powder or bovine serum albumin (BSA) in Tris-buffered saline with 0.1% (v/v) Tween 20 detergent (TTBS).

After blocking, the membranes were washed 3 times in 1xTTBS and incubated overnight at 4°C with continuous agitation in the primary antibody made up in 1xTTBS and blocking agent depending upon the protein of interest, as indicated in Table 3.3. Following overnight incubation, membranes were washed in 1xTTBS and incubated for

2 hours with horseradish peroxidase conjugated secondary antibodies; goat anti-rabbit or rabbit anti-mouse at a dilution of 1:1500 (Cell Signaling Technology, Danvers, MA, USA). Membranes were again washed 3 times in 1xTTBS and the bands were visualised using enhanced electrochemiluminescence (ECL) detection system (Thermo Scientific, Northumberland, UK) on the ChemiDoc™ Touch Imaging System (Bio-Rad, UK). Band intensities were measured using the associated Image Lab™ Touch Software and were normalised against total levels of GAPDH.

3.15.5 Processing pellet for the determination of 14kDa Actin fragment

The pellets produced during lysate preparation were removed from the freezer and suspended in 150µl of sample buffer without Bromophenol Blue, vortexed and heated at 100°C for 5 minutes, before being briefly sonicated. If necessary, samples were heated again and the step repeated until the pellet was back in solution. To measure the protein concentration of this solution a 50µl volume was removed to a new tube and mixed with an equal volume of 20% w/v Trichloroacetic Acid (TCA), vortexed and incubated at 4°C for 30 minutes. Following incubation, samples were microcentrifuged at 13,000rpm for 10 minutes at 4°C resulting in a pellet. The supernatant was aspirated and the pellet dissolved in 50µl 0.5M NaOH by placing the tubes in a water bath at 70°C for 30 minutes. The protein concentration was then determined by the Folin Lowry assay described above in section 3.16.3. After the determination of protein concentrations, the volume yielding 50µg of protein was taken from the original tube and combined with a small amount of Bromophenol Blue, before being loaded onto acrylamide gels following the procedures outlined above in section 3.16.4.

Table 3.4. Western blot antibodies and dilutions

Antibody	Source	Ref No	Dilution	Blocking Agent	Secondary Antibody
Phospho-Akt ^{Ser473}	Cell Signaling	4060	1:2000	5% Milk Powder	Rabbit
Phospho-P70S6K ^{Thr389}	Cell Signaling	9205	1:500	5% BSA	Rabbit
Phospho-mTOR ^{Ser2448}	Cell Signaling	5536	1:1000	5% BSA	Rabbit
Phospho-Smad2 $^{ser465/467}$/Smad3 $^{ser423/425}$	Cell Signaling	8828	1:1000	5% BSA	Rabbit
Total Smad2/3	Cell Signaling	8685	1:1000	5% BSA	Rabbit
Actin AC40 Clone	Sigma	A4700	1:500	5% Milk Powder	Mouse
GAPDH	Cell Signaling	5174	1:1000	5% Milk Powder	Rabbit

3.16 Statistical analysis

A primary analysis was performed for each variable using repeated measures ANOVA for biopsy time-point (baseline, 'untrained', and 'trained') and group allocation (AE and CE). Sphericity of the data was determined using Mauchly's test, and if violated, Greenhouse-Geisser correction was applied. The partial eta squared (ηp^2) effect size statistic was calculated for each dependent variable, and interpreted as: 0.01 small, 0.06 medium, and 0.14 large effect sizes (Bakeman 2005). In addition, paired t-tests were performed between specific time points (baseline - untrained, baseline - trained, and untrained - trained) following AE and CE to assess the effects of the different modes of exercise in the following unaccustomed and accustomed exercise.

A contemporary magnitude based inference (MBI) approach was used to investigate pre-post exercise differences between AE and CE and used to make inferences regarding the between group effects of the different exercise modalities at each biopsy time point. Using this approach, the mean effects of CE and their 90% confidence intervals (90% CI) were estimated using a published spreadsheet (Hopkins 2007) computed from changes in the variable of interest between biopsy time-points in the two groups, and adjusted for baseline values for each variable. The use of 90% CIs is suggested for MBI as they reasonably imply whether an outcome is clear, and if the true value of the outcome is very likely or unlikely to be negative or positive (Hopkins, Marshall et al. 2009). The spreadsheet also calculated quantitative and qualitative chances that the true effects were likely beneficial (increased), trivial, and harmful (decreased) when a Cohen's *d* effect size value of 0.2 was employed as the smallest meaningful change in outcomes between groups (Hopkins, Marshall et al. 2009, Hopkins 2007). Qualitative descriptors were then assigned to the quantitative percentile scores as follows: 25-75% *possible*, 75-95% *likely*, and >99% *most likely* (Batterham, Hopkins 2006). Effects were deemed unclear if there was a greater than 5% probability of being both substantially positive and substantially negative (Figure 3.4) (Hopkins, Marshall et al. 2009). Cohen's *d* effects sizes were interpreted as <0.2 'trivial', 0.2 'small', 0.5 'moderate' and >0.8 considered a 'large' (Cohen 1992).

Figure 3.4 Magnitude Based Inferences taken from Batterham and Hopkins (2006). MBI was used to make inferences regarding changes in gene and protein expression between aerobic and combined exercise. If the confidence interval includes both substantially positive (increased) and negative (decreased) values the effect is deemed unclear, whilst if the confidence intervals are substantial in one direction (negative or positive) the effect can be deemed positive or negative depending on the probability the effect will have the observed magnitude.

Chapter 4

Correlates of Skeletal Muscle Mass and Physical Function in non-dialysis CKD

4.1 Introduction

CKD is a catabolic condition characterised by the progressive loss of skeletal muscle tissue, the aetiology of which appears to be the result of a number of different underlying causes, including disease associated comorbidities in addition to its metabolic complications (acidosis, glucocorticoid production, abnormal protein metabolism and chronic inflammation) (Wang, Mitch 2014). Irrespective of the underlying aetiology, the loss of muscle mass is associated with poor outcomes, and is likely to have a grave impact on physical functioning (Johansen, Shubert et al. 2003).

Whilst the loss of skeletal muscle appears to be more prevalent and functionally severe in ESRD (McIntyre, Selby et al. 2006), evidence suggests that the wasting process starts early in the disease process (John, Sigrist et al. 2013, Clyne, Esbjörnsson et al. 1993, Heiwe, Clyne et al. 2005, Segura-Orti, Gordon et al. 2017). Moreover, recent observational studies have shown that the loss of muscle mass and physical dysfunction is associated with mortality and major cardiovascular events in non-dialysis dependent CKD patients (Pereira, Cordeiro et al. 2015, Harada, Suzuki et al. 2017). However the impact of muscle wasting and the association between measures of muscle mass and physical performance is not completely understood in the non-dialysis CKD population.

In addition to the loss of muscle mass, alterations in muscle composition are likely to occur as a result of CKD and contribute to skeletal muscle dysfunction. Models of CKD have demonstrated reduced ability of the skeletal muscle to repair and regenerate following injury that results in the accumulation of intramuscular fibrotic and adipose tissue (Dong, Dong et al. 2017). Furthermore, the accumulation of intramuscular adipose tissue is independently associated with physical performance and strength measures in maintenance haemodialysis patients (Cheema, Abas et al. 2010). However, the associations between changes in muscle composition as an indicator of muscle quality, and physical performance have not yet been investigated in non-dialysis CKD.

It is apparent that muscle dysfunction encompassing both structural and functional abnormalities is evident amongst CKD populations, and associates with poor outcomes. Consequently, it is important to be able to characterise and quantify these changes in individuals with CKD in order for early recognition of skeletal muscle dysfunction and allowing for the application of appropriate interventions aimed at improving muscle structure and function. However, the assessment of such domains is not without difficulties.

DXA is the most commonly used method to assess muscle mass, and this remains true within the kidney literature. This imaging method uses two X-ray beams of different energies (high and low) to estimate fat mass, lean body mass (indicative of muscle mass), and bone mineral density of the whole body, or of specific anatomical regions (e.g. arms, legs and trunk) using different equations. Furthermore, ALM calculated from the sum of the lean mass of the arms and legs, and the ratio of ALM and height in meters squared (ALMI) is currently the method of choice when diagnosing sarcopenia, with several cutoff points currently recommended for diagnosing sarcopenia (Chen, Liu et al. 2014, Cruz-Jentoft, Baeyens et al. 2010, Fearon, Strasser et al. 2011). However, the DXA instrument is not portable, requires a trained operator, and involves exposure to radiation, thus limiting its clinical applicability.

In contrast, BIA may offer a more practical alternative to DXA. BIA determines the impedance of an electrical current through the body tissues, working on the principle that lean body mass contains water and electrolytes, and is therefore a good electrical conductor, whereas fat mass and other tissues are poor conductors and offer resistance to the electrical current (Carrero, Johansen et al. 2016). Impedance variables are then placed into empirical equations along with the individual's characteristics (e.g. height, weight, age and sex) to provide estimates of body compartments.

Similar to DXA, the use of multi-compartmental BIA allows for the estimate of segmental lean mass (as described above) that is used to define low muscle mass. For these reasons, recent consensus statements for the diagnosis of sarcopenia and cachexia include BIA as

an option for the assessment of muscle mass (Chen, Liu et al. 2014, Cruz-Jentoft, Baeyens et al. 2010, Fearon, Strasser et al. 2011). BIA estimates of muscle mass are also present in the CKD literature. Recently, Pereiar and colleagues (2015) reported that sarcopenia defined as low BIA derived SMMI and handgrip strength was independently associated with mortality in NDD-CKD patients. However, the associations between indices of muscle mass derived from BIA and physical performance have not been investigated in this population.

Whilst both DXA and BIA allow for assessment of regional lean mass (i.e. lean mass in the arms or legs) they are unable to distinguish between individual muscle and the muscles within a specific region of interest (i.e. the quadriceps). This is particularly important in conditions such as CKD where lower extremity muscles may be more affected by the wasting process, and is likely to impact upon activities of daily living, such as walking, and rising from the chair. Moreover, evidence suggests that physical performance of the lower extremities is more closely associated with poor outcomes (Roshanravan, Robinson-Cohen et al. 2013). Therefore being able to assess specific muscle groups that are associated with physical performance may be of particular importance in CKD.

An alternative method of assessing muscle specific atrophy is by the implementation of imaging methods such as MRI, which is considered the gold standard for the assessment of muscle size (Reeves, Maganaris et al. 2004). Indeed, MRI measures of quadriceps CSA are associated with physical performance methods in dialysis (Johansen, Shubert et al. 2003) and more recently, non-dialysis CKD (Segura-Orti, Gordon et al. 2017). In addition, these methods allow for the assessment of qualitative changes in skeletal muscle, such as the accumulation of intramuscular adipose tissue which, as discussed above, affects muscular performance. Unfortunately, the use of MRI is limited predominantly to research settings due to the high cost, need for technical expertise required and time consuming image analysis.

Ultrasound (US) imaging is a commonly available technique used within the clinical setting, however its application for the assessment of muscle mass has been largely overlooked in comparison with the above methods. Despite this, US imaging allows for a quick assessment of individual muscle architectural characteristics, and has been shown to correlate well with the gold standard MRI (Worsley, Kitsell et al. 2014, Bemben 2002, Reeves, Maganaris et al. 2004). In addition to quantifying the architectural characteristics of specific muscles, US also provides an indication of muscle composition through assessment of muscle echo-intensity (EI). The assessment of EI works on the premise that lean tissue has low EI, whilst IMAT and increases in connective tissue within the muscle results in a high EI, thus the higher the EI, the 'lower quality' of the lean tissue. Measures of EI have been shown to correlate well with fibrous tissue assessed by serial biopsies, and are also associated with reduced physical performance amongst older populations (Rech, Radaelli et al. 2014, Wilhelm, Rech et al. 2014, Strasser, Draskovits et al. 2013, Watanabe, Yamada et al. 2013, Fukumoto, Ikezoe et al. 2012, Cadore, Izquierdo et al. 2012). However, despite its clinical applicability, to the best of my knowledge, there are no studies investigating ultrasound measures of the lower extremities and physical performance in CKD populations.

The purpose of the work presented in this chapter was to characterise the population taking part in the ExTra CKD study in terms of the prevalence of muscle wasting and to investigate possible associations between measures of muscle mass and quality, and physical performance amongst this cohort of non-dialysis CKD patients.

4.2 Methods

4.2.1 Study design and participant recruitment

This was a cross-sectional analysis of baseline outcomes form participants taking part in the ExTra CKD trial (ISRCTN registry registration number: 36489137) described in detail in Chapter 3.

4.2.2 Demographic, anthropometric and laboratory data

Patient demographics including age, gender, race, and comorbid conditions were obtained from a combination of self-report and/or data extraction from medical records. The participant's most recent laboratory reports of serum creatinine, albumin, haemoglobin were obtained from medical notes. eGFR was taken from hospital records and was calculated using the modified diet in renal disease equation, and was used to classify participants into CKD stages 3b-5 as per KDIGO guidelines. All other measures were obtained during specified study visits.

4.2.3 MRI derived quadriceps muscle volume

MRI is considered the gold standard for the assessment of muscle mass. Scans of participants' quadriceps were acquired in a 3T Siemens Skyra HD MRI scanner as described in section 3.6, and the volume of participants right quadriceps muscle was measured on 10mm thick slices by manually outlining the facial boundary using online software (Jim, Xinapse Systems, UK).

4.2.4 Ultrasound derived rectus femoris anatomical Cross-sectional area and echo intensity

Rectus femoris anatomical cross sectional area (RF-ACSA) and echo intensity (RF-EI) of participants' right leg was determined under resting conditions using B-mode 2D ultrasonography (Hitachi EUB-6500; probe frequency, 7.5 MHz) with the patient prone at a 45^0 angle (see section 3.5).

4.2.5 Bioelectrical impedance analysis

Whole body and segmental (right arm, left arm, trunk, right leg and left leg) lean and muscle mass were measured using 3-compartmental Direct Segmental Multi-frequency BIA (InBody370, InBody Bldg, Korea) as described in section 3.7. ALMI and SMMI

were calculated by the sum of lean mass in each arms and legs and total muscle mass divided by the participant's height in m^2, respectively. Low muscle mass was identified using cut-off values for ALMI of <7.26 kg/m^2 for men and <5.45 kg/m^2 for women (Baumgartner, Koehler et al. 1998) and SMMI of <10.26 kg/m^2 for men and <6.76 kg/m^2 for women (Cruz-Jentoft, Baeyens et al. 2010), both of which are recommended by the EWGSOP (2010) and have been used to diagnose sarcopenia in non-dialysis CKD populations (Zhou, Hellberg et al. 2017, Pereira, Cordeiro et al. 2015).

4.2.6 Muscular Strength

The maximal strength of the knee extensors was measured using a leg extension machine (Technogym, Cesen, Italy), using a 5-RM to estimate 1-RM (Brzycki 1993). The 5-RM protocol is described fully in section 3.8.

4.2.7 Cardiorespiratory Fitness

Peak oxygen consumption (VO_{2peak}) was determined during a symptom limited, incremental cycling test performed on an electrically braked cycle ergometer (Lode Excalibur Sport, Gronigen, the Netherlands) and breath-by breath sampling methods (Cortex Metalyzer, Leipzig, Germay) as described in section 3.9.

4.2.8 Exercise Capacity and walking performance

Exercise capacity and walking performance was assessed using the ISWT in accordance with the methods described in 3.10

4.2.9 Sit-to-stand tests

Physical function was determined using the sit-to-stand 5 (STS-5), a surrogate measure or muscular power and the sit-to-stand 60 (STS-60), a surrogate measure of muscular endurance (Koufaki, Mercer 2009) described in section 3.11.

4.2.10 Statistical analysis

All data was tested for normality using the Shapiro-Wilkes test. Associations between measures of muscle mass and physical performance were assessed using Pearson product-moment or Spearman's rank correlation coefficients accordingly. Multiple linear regression was used to determine the extent to which measures of muscle mass and size were associated with functional parameters. Because of the associations between age, gender, eGFR, and haemoglobin reported in the literature, these were included in the correlations and as covariates in the regression analyses. The results of the regression analyses are expressed as standardised coefficients of 1 standard deviation (SD) for each variable, where larger standardised coefficient means a greater contribution to the model. All statistical analysis was carried out using IBM SPSS Statistics (IBM, Chicago, IL) Version 24.

4.3 Results

4.3.1 Study population & missing data

Fifty-one patients (26 females) with CKD stages 3b-5 not requiring dialysis were included in this cross-sectional analysis. The mean age of patients was 61 ± 12 years old, with a mean eGFR of 24 ± 8 mL/min/1.73m^2. Full patient clinical and demographic characteristics are shown in Table 4.1. There was missing data for all variables other than age, eGFR, and Hb, therefore analysis was performed on the following number of patients for each of the following outcome measures; MRI (n=37, 73%), RF-CSA (n=46, 90%),

echo intensity (n=35, 69%), ALMI and SMMI (n=44, 86%), e1-RM (n=48, 94%), VO$_{2peak}$ (n=43, 84%), ISWT (n=49, 96%), STS-5 and -60 (n=49, 96%).

BIA was performed on 44 patients allowing for the calculation of ALMI and SMMI. When using ALMI, 7 patients (16%) were observed as having low muscle mass indicative of sarcopenia, which was more prevalent in women (4, 9%) than men (3, 6%) defined as ALMI <5.45 kg/m^2 and <7.26 kg/m^2, respectively (Baumgartner, Koehler et al. 1998). However, when using SMMI, 15 patients (34%) were classified as having low muscle mass: 2 (5%) women and 13 (30%) men defined as SMMI <6.76 kg/m^2 and <10.26 kg/m^2, respectively.

Table 4.1 Participant demographics

Characteristics	Total	Males	Females
Number of patients (n)	51	25	26
Age (y)	61 ± 12	63 ± 9	60 ± 14
Ethnicity (n):			
White	30	16	14
Indian/South Asian	18	8	10
Black Caribbean	3	1	2
BMI (kg/m^2)	29.6 ± 5.7	28 ± 4.5	31 ± 6.4
eGFR (mL/min/1.73m^2)	24 ± 8	23 ± 7	26 ± 8
Haemoglobin (g/L)	116 ± 14	121 ± 15	112 ± 12
Albumin (g/L)	41 ± 3	41 ± 2	41 ± 3
Comorbid conditions (n):			
Diabetes	16	9	7
CVD	9	6	3
Lupus	1	0	1
Muscle mass:			
[1]ALMI (Kg/m^2)	7.48 ± 1.25	8.02 ± .91	6.99 ± 1.33
[2]SMMI (Kg/m^2)	9.91 ± 1.56	10.41 ± 1.35	9.44 ± 1.63
Quadriceps vol. (cm^3)	961.5 ± 324.2	1114.2 ± 318.3	845.2 ± 283.4
RF-ACSA (cm^2)	8.3 ± 2.7	9.4 ± 2.5	7.3 ± 2.4
RF-EI, (a.u.)	72.5 ± 22.6	60.4 ± 13.	86.8 ± 23.3
Physical function:			
e1-RM (Kg)	47.0 ± 20.4	55.1 ± 22.6	39.0 ± 15.9
VO$_{2peak}$ (ml/kg/min)	19.5 ± 5.5	20.1 ± 6.5	18.7 ± 3.8
ISWT (m)	384.7 ± 183.8	416.7 ± 206.8	354.0 ± 156.9
STS-5 (sec)	15.48 ± 20.22	18.97 ± 28.4	12.14 ± 4.47
STS-60 (reps)	27 ± 13	27 ± 7	27 ± 13

Abbreviations: eGFR = estimated glomerular filtration rate; CVD = cardiovascular disease; ALMI = appendicular lean mass index; SMMI = skeletal muscle mass index; RF-ACSA = rectus femoris anatomical CSA; RF-EI = rectus femoris echo intensity; e1-RM = estimated 1-repetition maximum; VO$_{2peak}$ = maximal oxygen uptake; ISWT = incremental shuttle walking test; STS5 = sit-to-stand 5 test; STS60 = sit-to-stand 60.

Reference values for [1] ALMI <7.26 kg/m^2 for males and <5.45 kg/m^2 for females (Baumgartner, Koehler et al. 1998). [2] SMMI <10.26 kg/m^2 for males and <6.76 kg/m^2 for females (Cruz-Jentoft, Bayens et al. 2010).

4.3.2 Bivariate correlates of physical performance

The results from the bivariate correlations between measures of muscle quantity and quality, physical performance, and clinical variables can be found in Table 4.2. Of note, all measures of muscle mass were positively correlated with muscle strength (e1-RM), whereas only RF-CSA was correlated with all measures of physical performance. Interestingly, RF EI, which provides a measure of muscle quality was negatively associated with measures of muscle strength, VO_{2peak} and ISWT performance. In addition to muscle mass variables, age was associated with VO_{2peak}, ISWT, and physical performance (STS-5 and -60), but not muscle strength. In contrast, haemoglobin was associated with muscle strength, VO_{2peak} and ISWT only.

4.3.3 Multivariate linear regression

Correlates of muscle mass and performance variables were entered into linear regression models to determine the associations of muscle mass and size with physical performance, independent of clinical variables and co-morbidity. When accounting for eGFR, age, Hb, and the presence of diabetes, both ALMI (β=.497, p<.001) and SMMI (β=.418, p=.002) were independently associated with muscle strength. Similarly, both quadriceps volume and RF-CSA were independently associated with muscle strength, eliciting β values of β=.611 (p<.001) and β=.495 (p=.002), respectively. Interestingly, RF-CSA (β=.317, p=.034) and EI (β=-.293 p=.032) were also independently associated with VO_{2peak} but quadriceps volume was not (β=.174, p=.214). However, when accounting for the above-mentioned covariates, no measure of muscle mass or quality were independently associated with ISWT, STS-5 or -60 performance. The results of the multivariate linear regression analyses for muscle size can be found in Table 4.3.

In addition, the presence of low muscle mass defined using ALMI and SMMI were also entered into regression models. The presence of low muscle mass defined using ALMI cutoffs was independently associated with muscle strength (β=-.392, p=.003), but not when defined using SMMI cutoffs (β=.021, p=.867).

Table 4.2 Bivariate correlations between measures of muscle quantity and quality, physical performance and clinical parameters

Variable	Age	eGFR	Hb	Quadriceps Vol (cm³)	RF-CSA (cm²)	RF-EI (au)	ALMI (Kg/m²)	SMMI (Kg/m²)
Age	−	−	−	-.122	-.270	.300	.066	.201
eGFR	.093	−	−	.073	.212	.014	-.104	-.119
Hb	.114	**.284***	−	.305	**.579****	-.180	**.301***	.219
e1-RM [#]	-.216	-.103	**.494****	**.762****	**.719****	**-.351***	**.603****	**.435****
VO₂peak	**-.400****	.133	**.523****	**.352***	**.621****	**-.425***	-.011	-.135
ISWT	**-.419****	.001	**.435****	**.366***	**.461****	**-.347***	.187	0.96
STS-5	**.619****	.129	-.075	-.207	**-.349***	.239	.007	.064
STS-60 [#]	**-525****	-.057	.170	.174	**.719****	-.213	-.005	-.146

[#] Data presented as *rho* from Spearman's rank correlation. *Abbreviations*: eGFR = estimated glomerular filtration rate; Hb, = haemoglobin; e1-RM = estimated 1-repetition maximum; VO₂peak = maximal oxygen uptake; ISWT = incremental shuttle walking test; STS5 = sit-to-stand 5 test; STS60 = sit-to-stand 60. *p<.05 **p<.001

Table 4.3 Multivariate regression analysis between measures of muscle quality and quantity, with physical performance adjusted for eGFR, age, Hb, and diabetes

Dependent variable	Standardised coefficient, per 1 SD (*P*)				
	Quadriceps Vol	**RF-CSA**	**ALMI**	**SMMI**	**RF-EI**
e1-RM (kg)	**.611 (<.001)**	**.495 (.002)**	**.497 (<.001)**	**.418 (.002)**	-.224 (.193)
VO$_{2peak}$ (ml/kg/min)	.174 (.214)	**.316 (.038)**	–	–	**-.293 (.032)**
ISWT (m)	.205 (.188)	.124 (.446)	–	–	-.163 (.281)
STS-5 (secs)	–	.044 (.815)	–	–	–
STS-60 (reps)	–	.236 (.123)	–	–	–

Abbreviations: Quadriceps Vol = quadriceps volume measured by MRI; RF-CSA = rectus femoris CSA; ALMI = appendicular lean mass index; SMMI = skeletal muscle mass index; RF-EI = rectus femoris echo intensity; e1-RM = estimated 1-repetition maximum; VO$_{2peak}$ = maximal oxygen uptake; ISWT = incremental shuttle walking test; STS5 = sit-to-stand 5 test; STS60 = sit-to-stand 60.

4.4 Discussion

These cross-sectional analyses aimed to investigate the prevalence of low muscle mass, and the associations between muscle mass and size with physical performance in the cohort of non-dialysis CKD patients taking part in the ExTra CKD trial (described in Chapter 3). Amongst this cohort of patients with CKD stage 3b-5 not requiring dialysis, a prevalence of low muscle mass ranging from 16% to 34% was observed when defined by ALMI and SMMI cut-offs, respectively. This is higher than the prevalence previously reported in community-dwelling older people (mean age 67) in the UK of 4.6% in 765 men, and 7.9% in 1022 women (Patel, Syddall et al. 2013). However differences may attributable to the methods used to assess low muscle mass. Until recently very few studies had investigated the prevalence and consequences of muscle wasting in non-dialysis CKD. Pereira et al (2015) reported an increased risk of mortality in sarcopenic CKD patients amongst their cohort of 287 non-dialysis CKD participants on conservative care diagnosed using a combination of SMMI measured using BIA and handgrip strength cut-offs. The overall prevalence of 5% reported was lower than in our small cohort of CKD patients, which is likely due to the inclusion of handgrip strength as an index of muscle function into the diagnosis.

Zhou et al (2017) reported a higher prevalence of sarcopenia amongst a cohort of non-dialysis CKD patients when using DXA derived ALMI to define low muscle mass (36%), in comparison to a combination of ALMI and handgrip cut-offs (14%). Whilst sarcopenia is characterised by, and was originally defined by the involuntary loss of skeletal muscle, most consensus statements suggest the inclusion of muscle function or physical performance in its diagnosis (Cruz-Jentoft, Baeyens et al. 2010, Morley, Abbatecola et al. 2011, Fielding, Vellas et al. 2011). Therefore the present study is limited in classifying as having low muscle mass only. Although measures of functional performance are included in the present analysis, it was not possible to use these in the classification as no consensus of cut-off points currently exist for these measures.

The prevalence of low muscle mass reported in the present study and within the wider CKD literature changes depending upon the cut-off points used to define the condition. In support of this, Souza et al (2017) recently reported the prevalence ranging from 12% to 29% when using ALMI cut-off points from EWGSOP (2010) and BMI adjusted lean mass cut-offs suggested by the Foundation for the National Institutes of Health (2014). Irrespective of the criteria used to identify sarcopenia, it appears that the loss of muscle mass is of clinical importance in CKD patients, and is associated with increased risk of mortality (Pereira, Cordeiro et al. 2015) and major cardiovascular events (Harada, Suzuki et al. 2017). Therefore more research is needed to not only identify the prevalence of sarcopenia in non-dialysis CKD populations, but best cut-off points to identify individuals with low muscle mass and interventions capable of increasing muscle mass and function.

In addition to muscle wasting, poor physical performance, particularly of the lower extremities is associated with poor outcomes amongst non-dialysis CKD (Roshanravan, Robinson-Cohen et al. 2013). For perspective, the physical performance measures reported in this cohort of non-dialysis CKD patients is similar in terms of exercise capacity measured as VO_{2peak} (Faria Rde, Fernandes et al. 2013, Aoike, Baria et al. 2017, Downey, Liao et al. 2017, Headley, Germain et al. 2017, Van Craenenbroeck, Van Craenenbroeck et al. 2016, Greenwood, Koufaki et al. 2015) and ISWT as previously reported in non-dialysis CKD patients (Watson, Greening et al. 2014). However, we observed better STS-5 and -60 scores, and a larger mean RF-ACSA (Watson, Greening et al. 2014) than previously reported by our group. Although the loss of muscle mass is frequently reported as a likely cause of reduced physical performance, there is little evidence for the association between measures of muscle mass and individual muscle size with physical performance in this population.

Amongst this cohort of non-dialysis CKD participants, strong associations were observed between measures of muscle mass and size and physical performance. Both ALMI and SMMI were positively and independently associated with muscle strength, but not other measures of physical performance. Furthermore, when accounting for eGFR, age, gender, and, comorbidities, the presence of muscle wasting defined using ALMI cut-offs was

independently associated with muscle strength, but not when defined as low SMMI. The fact that ALMI accounts for the muscle mass within the extremities, it is not surprising that low muscle mass defined using these cut-offs is associated with physical performance of the lower extremities. Indeed, in their study, Zhou et al (2017) reported that limb lean mass was independently associated with both upper and lower extremity strength in non-dialysis CKD, but did not report whether the presence of low muscle mass itself was associated with muscle function, whilst Souza et al (2017) reported that sarcopenia defined as ALM adjusted for BMI was independently associated with gait speed amongst their cohort of CKD patient's stages 2-5.

In contrast to BIA measures of muscle mass, strong associations between muscle size and physical performance were observed. One of the main drawbacks to the use of DXA or BIA derived cut-offs is the inability to identify specific muscles or muscle groups that may be prone to muscle wasting, and/or are important for physical performance. Whilst atrophy of the non-locomotor muscles is observed in ESRD patients (Sakkas, Ball et al. 2003) evidence suggests that lower limb muscles, particularly the quadriceps are affected. The use of imaging techniques such as MRI and US adds information beyond total or appendicular muscle mass, allowing a more precise investigation of a region of interest (such as specific muscle groups or muscles as in the present study) and how it associates with physical performance.

Indeed, measures of quadriceps muscle volume and RF-ACSA were more strongly associated with lower extremity muscle strength than those of ALMI or SMMI (Table 4.3). Furthermore, multivariate linear regression revealed that both quad volume and RF-ACSA were independently associated with knee extensor strength. We also observed positive correlations between both quadriceps volume and RF-ACSA with VO_{2peak} and ISWT performance, however, when controlling for covariates, only RF-ACSA was independently associated with VO_{2peak}. Similarly, in a recent study Segura-Orti (2017) also reported independent associations between quadriceps muscle group CSA, muscle strength and exercise capacity measured using 6-minute walk test, amongst a cohort of 22 CKD patients stage 3-4. Taken together these suggest a possible link between muscle

atrophy, physical performance and exercise capacity, which is also independently associated with poor outcomes in CKD populations (Sietsema, Amato et al. 2004).

In addition to measures of muscle mass and size, we also observed negative associations between RF EI, muscle strength and exercise capacity (VO_{2peak} and ISWT). When controlling for possible covariates, EI remained independently associated with VO_{2peak}. Echo intensity provides a measure of muscle quality based on a grey scale analysis, where a higher echo intensity may indicate increased intramuscular adipose tissue and/or fibrotic connective tissue (Young, Jenkins et al. 2015, Pillen, Tak et al. 2009). Indeed, a number of studies have demonstrated negative associations between echo intensity, muscle function, and cardiovascular performance in elderly participants (Rech, Radaelli et al. 2014, Wilhelm, Rech et al. 2014, Strasser, Draskovits et al. 2013, Watanabe, Yamada et al. 2013, Fukumoto, Ikezoe et al. 2012, Cadore, Izquierdo et al. 2012). To the best of my knowledge, this is the first investigation of skeletal muscle echo intensity and physical performance in non-dialysis CKD populations, however others have reported altered muscle composition through increased intramuscular adipose tissue was associated with worse physical performance amongst haemodialysis (Cheema, Abas et al. 2010).

4.5 Limitations

In order to accurately interpret the results of the present analysis, it is important to recognise the number of limitations. The cross-sectional nature of the study prevents the determination of directional relationships and causality of the observed associations between muscle mass, size and physical performance. Furthermore, the analysis was performed on a relatively small sample size, which was further reduced due to missing data, particularly for MRI and BIA assessments. In addition, there was insufficient data to include further clinical parameters (serum bicarbonate, uraemic toxins, and iron status) or measures of physical activity, that are potential mediators or confounders of the association between muscle mass and physical performance in CKD populations.

4.6 Conclusion

In conclusion, indices of muscle mass and individual muscle size correlated with muscle strength, exercise capacity and physical functioning amongst non-dialysis CKD patients. Furthermore, the presence of muscle wasting, quadriceps volume and RF-ACSA were independently associated with muscle strength, whilst RF-ACSA was also independently associated with VO_{2peak}. Taken together, these results suggest that muscle wasting and atrophy that are commonly reported in CKD populations may affect physical functioning in non-dialysis CKD patients. These independent associations suggest that interventions to increase muscle size and mass may be beneficial for CKD patients not yet requiring dialysis. This highlights the importance of interventions, such as exercise that are capable of increasing both muscle mass and function in this population. However, more research is needed to determine the optimal mode, frequency, and intensity of exercise training to elicit improvements in muscle mass, function and physical performance.

Chapter 5

A Randomised Trial on the Effects of 12-weeks Aerobic or Combined Exercise on Muscle Strength and Size in non-dialysis CKD

5.1 Introduction

Patients with CKD exhibit skeletal muscle wasting, in addition to reductions in muscular strength, physical function and cardiorespiratory fitness, which ultimately results in elevated cardiovascular risk. Whilst the loss of muscle mass may be more prevalent in those receiving haemodialysis, evidence suggests that the wasting process starts early in the disease progression (John, Sigrist et al. 2013, Heiwe, Clyne et al. 2005, Clyne, Esbjörnsson et al. 1993)

Reductions in muscle mass has a significant impact on physical functioning (Johansen, Shubert et al. 2003, Segura-Orti, Gordon et al. 2017), and is thought to be one of the main determinants of exercise tolerance in CKD populations (Diesel, Noakes et al. 1990). Moreover, both muscle wasting and physical dysfunction have important clinical implications in the non-dialysis CKD population. Indeed, a number of observational studies have demonstrated strong relationships between reductions in physical performance and muscle mass with greater risk of mortality (Pereira, Cordeiro et al. 2015, Roshanravan, Robinson-Cohen et al. 2013) and major adverse cardiovascular events (Harada, Suzuki et al. 2017), making them important modifiable targets for intervention.

Exercise is an intervention that can beneficially impact upon both muscle mass and physical performance in CKD populations (discussed in Chapter 2) and in recent years, exercise has started to be included in clinical guidelines for the treatment and management of CKD (KIDGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. 2013, K/DOQI Clinical Practice Guidelines for Cardiovascular Disease in Dialysis Patients. 2005). Despite this, and the growing evidence to support the inclusion of exercise rehabilitation in kidney disease populations, the provision of exercise advice and rehabilitation programmes in the UK are well behind that of other chronic conditions. This is likely due to a lack of specific guidance on the optimal modalities of exercise prescription and how these should be implemented.

In non-dialysis CKD, aerobic exercise has been shown to produce cardiovascular benefits for patients such as improvements in aerobic capacity, vascular stiffness, blood pressure

control, and anti-inflammatory effects (Heiwe, Jacobson 2011, Heiwe, Jacobson 2014, Viana, Kosmadakis et al. 2014). However, its effects on skeletal muscle remain equivocal. Furthermore, previous work from our group suggests it may have important negative effects on skeletal muscle in CKD patients that may limit the ability to increase muscle mass (Watson, Kosmadakis et al. 2013). On the other hand, progressive resistance exercise can increase muscle size, muscle strength and physical function in CKD populations (Cheema, Chan et al. 2014, Watson, Greening et al. 2014).

Both aerobic and resistance exercise appear to confer important benefits for individuals with CKD; as a result recent position stand points recommended that patients perform a combination of both aerobic and resistance exercise (Smart, Williams et al. 2013). However, whilst a handful of studies have investigated the effects of combined exercise programmes incorporating a combination of aerobic and resistance exercise, reporting improvements in exercise capacity and physical performance in comparison to non-exercise control subjects (Headley, Germain et al. 2012, Greenwood, Lindup et al. 2012, Clyne, Ekholm et al. 1991) a recent systematic review and meta-analysis concluded that combined exercise programmes require further research in view of their potential to impact upon a number of health outcomes (Heiwe, Jacobson 2014). In support of this, only one study has reported improvements in thigh muscle function following a combined exercise programme (Clyne, Ekholm et al. 1991) and no studies have investigated the effects of combined exercise on muscle hypertrophy in non-dialysis CKD patients.

Therefore, this chapter aimed to investigate if 12-weeks of combined aerobic and resistance exercise would result in greater increases in muscle strength and size, than performing aerobic training alone in patients with non-dialysis CKD. A secondary aim was to investigate how increases in muscle size following exercise relate to improvements in physical function.

5.2 Methods

This chapter describes the results of the ExTra CKD trial (ISRCTN registry registration number: 36489137) described fully in Chapter 3.

5.3 Primary outcome

5.3.1 Muscular Strength

The study was powered to detect a change in total weight lifted over the 12-week duration of the study in participants performing CE, and was calculated as weight x repetitions x sets. Maximal knee extensor strength was measured using a 5-RM protocol on a leg extension machine (Technogym, Cesen, Italy) as previously described (section 3.8). Estimated 1-RM was then calculated using the equation by (Brzycki 1993).

5.4 Secondary outcomes

5.4.1 MRI derived quadriceps muscle volume

Images of the entire quadriceps were acquired in a 3T Siemens Skyra HD MRI scanner using the parameters described in section 3.6. The volume of participants right quadriceps muscle was measured on 10mm thick slices using Jim online imaging analysis software (Xinapse Systems, UK).

5.4.2 Ultrasound derived rectus femoris anatomical Cross-sectional area

Anatomical cross sectional area of rectus femoris (RF-CSA) of the right leg was determined under resting conditions using B-mode 2D ultrasonography (Hitachi EUB-6500; probe frequency, 7.5 MHz) with the patient prone at a 45^0 angle (see section 3.5).

5.4.3 Body Composition

Whole body and segmental (right arm, left arm, trunk, right leg and left leg) lean and fat mass were measured using multi-frequency bioelectrical impedance analysis (InBody370, InBody Bldg, Korea) in accordance with the manufacturers instructions (see section 3.7). ALMI was calculated as previously described in section 4.2.5.

5.4.4 Cardiorespiratory Fitness

VO_{2peak} was determined during a symptom limited, incremental cycling test performed on an electrically braked cycle ergometer (Lode Excalibur Sport, Gronigen, the Netherlands) using breath-by breath sampling methods (Cortex Metalyzer, Leipzig, Germay) as described previously (section 3.9).

5.4.5 Exercise Capacity

Exercise capacity and walking performance was assessed using the ISWT (Singh, Morgan et al. 1992) as previously described (section 3.10).

5.4.6 Sit-to-stand tests

Physical function was determined using the STS-5, a surrogate measure or muscular power and STS-60, a surrogate measure of muscular endurance (Koufaki, Mercer 2009). The tests were carried out as described in section 3.11.

5.5 Statistical Analysis

All data was tested for normality using the Shapiro-Wilkes test. If data was not normally distributed, analysis was performed on the log-transformed data. The six-week control

period was analysed separately as this was prior to randomisation. Paired samples t-tests were used to determine differences between assessments before (baseline 1) and after (baseline 2) the 6-week control period. Intraclass correlations (ICC) (*r*) were used to determine the test-retest relative reliability of the data to investigate if any within subject changes occurred. An ICC between .600-.749 is considered 'fair', .750-.899 'good', and ≥ 900 considered excellent for clinical measures (Cicchetti 1994)

Exercise data was analysed using paired samples t-tests to determine the within group changes following each intervention. Linear regression models were fitted to determine the differences between the two groups with the change in outcome measure as the dependent variable, and the group assignment, age, gender, haemoglobin, diabetes status, and baseline value as the covariates. All data is presented as mean ± standard deviation and mean change with 95% confidence intervals (CI) unless stated otherwise. Regression modelling was also used to determine the contribution of increase in muscle size to outcome measures of interest. No adjustments for multiple comparisons were made in the analyses. All statistical analysis was carried out using IBM SPSS Statistics (IBM, Chicago, IL) Version 24.

5.5.1 Missing data

Missing data was analysed using Little's test, to test the assumption of missing completely at random (MCAR). Under the assumption that missing data was MCAR complete case analysis was performed, as although this reduces the power of the study it does not bias the results (White, Carpenter et al. 2012). A sensitivity analysis was also performed using intention-to-treat methods, whereby missing data from participants who had been randomised was imputed using last-observation-carried forward to confirm the results from the complete case analysis with the restored sample size (Thabane, Mbuagbaw et al. 2013). There was complete agreement with all variables analysed using the two methods, therefore only data from the complete case analysis are presented.

5.6 Results

5.6.1 Protocol adherence

Of the 54 patients recruited, 41 were randomised to receive 12-weeks of either AE (n=21) or CE (n=20). The flow of participants through the study is shown in Figure 5.1. Subsequently, five participants withdrew from the study during the intervention period, due to unrelated injury, development of another illness, or progression to ESRD, leaving 36 participants who completed the exercise intervention (n=18 in each group). A breakdown of participants' baseline characteristics can be found in Table 5.1. Mean adherence rate to the exercise sessions was 88% ± 13 for the AE group, and 88% ± 9 for CE. On average both groups met the target intensity of 70-80% of HRmax with mean values of 77% ± 8 and 75% ± 6 for the AE and CE groups, respectively. Both groups had a mean RPE for exercise sessions of 13 (target 12-14).

5.6.2 Non-exercise control data

Minimal differences were seen over the 6-week non-exercise control period for muscle mass measured as quadriceps volume, RF-CSA, and ALMI (Table 5.2). Furthermore, ICC r values of .986, .955, .935 for quadriceps volume, RF-CSA and ALMI, respectively suggest 'excellent' agreement between individual scores. Similarly, minimal changes were observed for VO_{2peak}, ISWT, and STS-60, with 'excellent' (ISWT) and 'good' (VO_{2peak} and STS-60) agreement. Conversely, a statistically significant increase of 2.9kg was observed for e1-RM over the control period, despite an 'excellent' ICC r value of .941. Whilst a mean increase of 1.9 seconds in STS-5 performance occurred over this period, with an ICC value representing only 'fair' agreement between scores (r=.560).

Table 5.1. Patient demographics at baseline. Data presented as median [IQR].

	Randomised AE	Randomised CE	Completed AE	Completed CE
Number of participants	21	20	18	18
Number of Males	9	9	7	7
Age (y)	63 [58-71]	63 [51-69]	63 [57-71]	63 [50-70]
Ethnicity (n)				
White	15	11	13	10
Indian/South Asian	4	9	4	8
Black Caribbean	2	0	1	0
BMI (kg/m^2)	29 [25.5-35.5]	29.5 [25.5-33.0]	29 [25.4-33.2]	29.6 [25.8-32.4]
ALMI (kg/m^2)	7.91 [6.47-8.37]	7.53 [7.24-7.92]	7.47 [5.95-8.40]	7.46 [6.87-7.99]
eGFR (mL/min/1.73m^2)	24 [20-30]	27 [22-32]	24 [20-35]	26 [19-30]
Systolic blood pressure (mm Hg)	135 [122-145]	125 [120-132]	146 [131-155]	124 [120-132]
Diastolic blood pressure (mm Hg)	73 [68-81]	70 [72-78]	72 [70-73]	70 [60-74]
Haemoglobin (g/L)	119 [115-131]	112 [105.5-128.5]	120 [115-132]	112 [106-125]
Albumin (g/L)	42 [41-44]	40.5 [38.5-42]	41 [39-42]	40 [37-42]
Serum total cholesterol (mmol/L)	4.4 [3.7-4.8]	3.6 [3.5-3.9]	4.4 [3.8-4.4]	3.6 [3.5-3.9]
Serum total triglycerides (mmol/L)	1.6 [1.2-2.2]	1.3 [1.0-2.1]	2.3 [1.9-2.5]	1.5 [1.0-2.1]
Leukocyte count (x10^9/L)	7.8 [6.8-8.3]	7.3 [6.5-8.8]	7.2 [6.5-9.2]	7.4 [6.7-7.8]
Haemoglobin A$_{1c}$ (%)	6.3 [5.6-7.5]	5.8 [5.5-5.9]	6.3 [5.6-7.5]	5.8 [5.5-5.9]
Comorbid conditions				
Diabetes (n)	8	4	7	2
CVD (n)	4	4	3	3
Lupus (n)	0	1	0	1

Figure 5.1 CONSORT diagram showing flow of patients through the study

Table 5.2 Mean change and relative reliability of outcome measures following 6-weeks non-exercise control period (*n*=36)

Variable	Baseline 1	Baseline 2	Difference (95%CI)	*P*	ICC (*r*)	ICC 95% CI (*r*)
Quadriceps Vol (cm^3)	949.3 ± 320.7	935.5 ± 315.5	-13.8 (-5.6 to 33.2)	.157	.986*	.972 to .997
RF-ACSA (cm^2)	8.47 ± 2.61	8.67 ± 2.87	.20 (-.50 to .11)	.200	.955*	.952 to .989
ALMI (kg/m^2)	7.24 ± 1.23	7.26 ± 1.29	+.02 (-.19 to .15)	.807	.935*	.867 to .968
e1-RM (kg)	47.4 ± 21.5	50.3 ± 22.6	+2.9 (-5.6 to -.17)	.038	.941*	.882 to .971
VO$_{2peak}$ (ml/kg/min)	20.1 ± 5.3	20.4 ± 5.6	+.3 (-1.1 to .63)	.808	.896*	.798 to .948
Watt Max (W)	119 ± 42	118 ± 41	-1 (-4 to 7)	.685	.931*	.863 to .966
ISWT (m)	407 ± 194	418 ± 195	+11 (-26 to 5)	.167	.974*	.949 to .987
STS-5 (secs)	12.4 ± 8.1	10.5 ± 4.4	-1.9 (-2.9 to .70)	.082	.560*	.273 to .755
STS-60 (reps)	29 ± 14	30 ± 14	+1 (-3 to 1)	.223	.935*	.873 to .968

Abbreviations: Quadriceps Vol = quadriceps volume; RF-CSA = rectus femoris cross-sectional area; ALMI = appendicular lean mass index; e1-RM = estimated 1-repetition maximum; VO$_{2peak}$ = peak oxygen uptake; ISWT = incremental shuttle walking test; STS-5 = sit-to-stand 5 test; STS-60 = sit-to-stand 60.

*All ICC *p*<.001

5.6.3 Muscle strength

Total weight lifted in a single exercise session in those performing CE increased by a mean of 648.1 (CI 390.9 to 905.4, $p<.001$) over the duration of the intervention. There was missing data for estimated 1-RM from one participant at baseline, meaning a complete case analysis was performed on 35 patients (AE n=18 and CE n=17). Increases in muscle strength measured as e1-RM were observed in both groups, with mean changes of 9.2kg (CI 4.6 to 13.9kg, $p<.001$) and 22.2kg (CI 15.6 to 28.7kg, $p<.001$) following AE and CE respectively (Figure 5.2). Linear regression revealed the increase in estimated 1-RM was greater in those performing CE by a mean of 12.9kg (CI 5.3 to 20.5, $p=.020$), which remained when accounting for the presence of diabetes, haemoglobin, age, gender and baseline strength. The increase in muscle strength was not explained by increases in RF-CSA ($R^2=.038$, $p=.257$) or quadriceps volume ($R^2=.077$, $p=.130$).

5.6.4 Quadriceps Volume measured by MRI

Quadriceps volume data was available for 31 participants (AE n=15 and CE n=16) who completed the intervention. Reasons for the missing data include; patients exhibiting contraindications to MRI procedures (n=2), having joint replacements that would affect scan procedure and analysis (n=2), and opting out of the procedure (n=1). Quadriceps volume was increased in both groups following 12-weeks regular exercise. A mean increase of 40.5cm^3 (CI 2.7 to 78.3cm^3, $p=.037$) was seen following AE, compared to a mean increase of 88.0cm^3 (47.6 – 128.5cm^3, $p<.001$) seen in those who performed CE, corresponding to increases of 4.3% and 9.4%, respectively. When accounting for differences in the presence of diabetes, haemoglobin, age, gender and baseline quadriceps volume, the magnitude of change was 47.5cm^3 (CI -5.7 – 100.7cm^3, $p=.012$) greater in those performing CE. Figure 5.2 shows the increase in quadriceps volume following 12-weeks of AE and CE.

Table 5.3 The effects of aerobic or combined exercise on muscle strength and hypertrophy

Variable	Aerobic	Combined	Difference (95% CI)	P*
e1-RM (kg)				
Baseline	54.3 ± 26.6	45.5 ± 16.1		
Post exercise	63.5 ± 25.6	67.7 ± 22.2	12.9 (5.3 to 20.5)	**.020**
Change	9.2 (4.6 to 13.9)	22.2 (15.6 to 28.7)		
P	<.001	<.001		
Quadriceps Vol (cm³)				
Baseline	939.0 ± 344.8	932.2 ± 296.9	47.5	**.012**
Post exercise	979.5 ± 355.5	1020.2 ± 329.0	(-5.7 to 100.7)	
Change	40.5 (2.7 to 78.3)	88.0 (47.6 to 78.3)		
P	**.037**	**<.001**		
RF-ACSA (cm²)				
Baseline	8.7 ± 3.1	8.3 ± 2.7		
Post exercise	9.0 ± 3.2	9.0 ± 2.7	.4 (-.1 to .9)	.371
Change	0.4 (-.1 to .9)	.7 (.4 to 1.1)		
P	.126	**<.001**		

$P*$ adjusted for Age, Gender, baseline values, presence of diabetes and Hb at baseline. *Abbreviations:* Quadriceps Vol = quadriceps volume; RF-CSA = rectus femoris cross-sectional area; ALMI = appendicular lean mass index.

A

B

Figure 5.2 Change in knee extensor strength (A) and quadriceps volume (B) following 12-weeks aerobic or combined exercise. *_p_<.05 from baseline; #_p_<.05 from aerobic exercise

5.6.5   Rectus Femoris Anatomical CSA

There was missing data for one of the 36 participants that completed the exercise intervention, therefore RF-ACSA was analysed for a total of 35 participants following AE (n=17) and CE (n=18). There were mean increases in RF-ACSA of .4cm^2 (CI -.1 to .9cm^2, _p_=.126) following 12-weeks of AE compared to a mean increase of .7cm^2 (CI .4 to 1.1cm^2, _p_<.001) following CE. This corresponds to mean percentage increases of 3.5% and 8.8% following AE and CE, respectively. Despite the mean change of .4cm^2 (CI -.1 to .9cm^3) between groups this was not statistically significant (_p_=.498).

5.6.6 Body composition and muscle mass

Body composition analysis was available for 31 patients who completed the exercise intervention period (AE n=15 and CE n=16). Small mean changes were observed in body mass following 12-weeks of supervised exercise for both groups (AE p=.522 [-0.5kg, CI -1.9 to 1.0kg]; CE p=.538, [-0.2kg CI -1.0 to .6kg]). Similarly, only small mean changes were seen for total skeletal muscle mass kg (SMM) and ALMI (kg/m^2) measured by BIA, with mean change of 0.2 kg for SMM (p=.556 [CI -0.4 to 0.7 kg]) and 0.1 for ALMI (p=.551 [-0.2 to $0.3kg/m^2$]) following AE. Following CE mean changes of -0.22kg were observed for SMM (p=.657 [CI -1.3 to 0.8kg]) and -0.04 kg/m^2 (p=.806 [-0.35 to 0.27 kg/m^2]) for ALMI. No between group differences were seen for any of the body composition variables (BM p=.973 [difference -0.03kg, CI -1.773 to 1.714]; SMM p=.630 [difference -0.3kg, CI -1.331 to .821]; and ALMI p=.918 [difference -1.49kg/m^2 CI -6.54 to 3.56]).

5.6.7 Cardiorespiratory fitness (VO$_2$peak)

Out of the 36 completers, VO$_{2peak}$ data was available for 32 participants (AE n=15 and CE n=17). Mean changes in absolute VO$_{2peak}$ were .06 L/min (-.05 to .17 L/min, p=.256]) following AE, and .02 L/min (CI -.12 to .16 L/min, p=.753) following CE. Similarly, relative VO$_{2peak}$ exhibited small mean changes of 1.07 ml/kg/min (CI -.29 to 2.43 ml/kg/min, p=.113) and .63 ml/kg/min (CI -1.23 to 2.48 ml/kg/min, p=.483) following AE and CE respectively. No statistical group differences were observed in the regression model for absolute (p=.138 [difference -.131 L/min CI -.31 to .05]) and relative (p=.911 [difference -.44 ml/kg/min CI -2.70 to 1.82]) VO$_{2peak}$.

5.6.8 Peak power output

Peak power output measured as maximum wattage (W) achieved during the graded exercise test (i.e. at VO$_{2peak}$) was available for 32 participants (AE n=15 and CE n=17) who completed the intervention. Both AE and CE groups exhibited similar mean increases of 8.7 W (CI -2.0 to 19.4 W, p=.102) and 8.0 W (CI .3 to 16.0 W, p=.043),

respectively. No statistical differences were observed between exercise modes (difference -.7 CI -13.0 to 11.7, *p*=.302).

5.6.9 Walking performance and exercise capacity measured by an incremental shuttle-walking test

The ISWT was used as a measure of participants walking performance and a second measure of exercise capacity by recording the distance walked in meters (m) during the externally paced test. A complete case analysis was performed on 35 patients (AE n=18 and CE n=17) revealing mean increases of 28m (CI 6 to 50m, *p*=.010) and 32m (CI 9 to 56m, *p*=.010) following AE and CE respectively. No statistical difference between groups was seen (difference 5m CI -26 to 34, *p*=.982).

5.6.10 Sit-to-stand 5 (STS-5) and Sit-to-stand 60 (STS-60)

The STS-5 and STS-60 were included as surrogate measures of lower extremity muscular strength and endurance respectively. Complete case analysis was performed for all 36 participants who completed the 12-week exercise intervention (n=18 in each group). STS-5 scores exhibited mean reductions in the time to complete the test of -1.5 seconds (CI -2.7 to -0.5 seconds, *p*=.004) following AE and -1.1 seconds (CI -3.2 to 1.1 seconds, *p*=.290) following CE. Similarly, STS-60 scores showed mean increases in the number of repetitions performed in 60 seconds for both groups, with mean increases of 10 (CI -2 to 21 reps, *p*=.102) and 6 reps (CI 3 to 8 reps, *p*=.002) following AE and CE respectively. The change in STS scores tended to be greater following AE, however, no statistical differences were noted between groups for either STS-5 (difference .5 seconds CI -1.8 to 2.8, *p*=.373) or STS-60 (difference -4 reps CI -17 to 8, *p*=.819).

Table 5.4 The effects of 12-weeks aerobic or combined exercise on physical function

Variable	Aerobic	Combined	Difference (95% CI)	P*
VO$_{2peak}$ (ml/kg/min)				
Baseline	21.43 ± 6.42	19.48 ± 4.73		
Post exercise	22.50 ± 6.60	20.12 ± 4.96	-.44	.911
Change	1.07 (-.29 to 2.43)	.63 (-1.23 to 2.48)	(-2.70 to 1.82)	
P	.113	.483		
Max Wattage (W)				
Baseline	129.0 ± 48.0	110.6 ± 32.2		
Post exercise	137.7 ± 41.6	118.6 ± 36.6	-.7	.302
Change	8.7 (-2.0 to 19.4)	8.0 (.3 to 15.7)	(-13.0 to 11.7)	
P	.102	.043		
ISWT (m)				
Baseline	454 ± 194	380 ± 195		
Post exercise	482 ± 190	412 ± 195	5 (-26 to 36)	.982
Change	28 (6 to 50)	32 (9 to 56)		
P	.010	.010		
STS-5 (secs)				
Baseline	10.3 ± 3.2	10.60 ± 5.32		
Post exercise	8.7 ± 2.7	9.60 ± 6.23	.5 (-1.8 to 2.8)	.693
Change	-1.5 (-2.7 to -.5)	-1.1 (-3.2 to 1.1)		
P	.004	.290		
STS-60 (reps)				
Baseline	31 ± 14	28 ± 14		
Post exercise	42 ± 28	34 ± 14	-4 (-17 to 8)	.819
Change	10 (-2 to 22)	6 (3 to 8)		
P	.102	.002		

P adjusted for Age, Gender, baseline values, presence of diabetes and Hb at baseline. *Abbreviations:* e1-RM = estimated 1-repetition maximum; VO$_{2peak}$ = peak oxygen uptake; ISWT = incremental shuttle walking test; STS-5 = sit-to-stand 5 test; STS-60 = sit-to-stand 60.

5.7 Discussion

Evidence for the benefits of exercise in the non-dialysis CKD population is growing, however the majority of trials have investigated the effects of exercise in comparison to a control group. Research now needs to focus on finding the best exercise prescription for health benefits in this population in order to make specific exercise recommendations. Improvements in skeletal muscle mass and function are two important outcomes following exercise training in CKD populations, however the effects of aerobic exercise and a combination of aerobic and resistance exercise on skeletal muscle properties have not been fully investigated in non-dialysis CKD. To the best of my knowledge, this is the first study to directly compare two separate exercise prescriptions on skeletal muscle mass and function in non-dialysis CKD patients.

5.7.1 Non-exercise control period

Over the 6-week non-exercise control period only small mean changes and 'good' or 'excellent' agreement between the two scores for outcome measures of muscle mass (quadriceps volume, RF-CSA, and ALMI) and physical performance (ISWT, VO_{2peak}, and STS-60) suggesting no change in these indices. This is in line with other studies in non-dialysis CKD patients who have reported no change in muscle mass or physical performance in participants randomised to non-exercise control groups (Watson, Greening et al. 2014, Castaneda, Gordon et al. 2001).

In contrast, an increase of 2.9kg was observed for estimated 1RM over this period, despite showing 'excellent' agreement. This could represent a learning effect following the initial testing, however, familiarisation tests were conducted on a separate visit prior to the initial (baseline 1) testing being conducted in order to minimise the possibility of any such effect. Moreover, the minimum increase in weight lifted during the 5-RM protocol was 2.5kg, therefore this may simply represent an artefact due to the limitations of the machine. Similarly, we observed a mean of 1.9 second improvement in STS-5 performance over the control period. Again, familiarisations were performed prior to the initial testing, however, this change observed may partly be attributed to the poor

reliability of the test. Indeed, only a 'fair' ICC score was observed for the STS-5, which may indicate a large within-patient variability.

5.7.2   Increases in muscle strength following exercise

Both groups exhibited marked improvements in muscle strength with mean increases in e1-RM by 9.2kg and 22.2kg following AE and CE respectively. The superior gains in strength seen in those performing CE is not surprising given the specificity of the resistance exercise in those performing CE. Indeed, the increase of 22.2kg following CE corresponding to 49% is similar to that previously reported following progressive resistance exercise only interventions in non-dialysis CKD where improvements in knee extensor strength range from 11-47% (Watson, Greening et al. 2014, Heiwe, Tollback et al. 2001, Castaneda, Gordon et al. 2001). However, whilst lower limb muscle function is associated with important outcomes in non-dialysis CKD participants, future studies are required to investigate the clinical importance of increases in lower limb muscle strength following exercise in this population.

Despite the close association between muscle size and strength observed in Chapter 4, no association was found between the increased strength and changes in changes in quadriceps muscle size, with the change in RF-ACSA and quadriceps volume accounting for only 3.8% and 7.7%, of the variance in the increased e1-RM, respectively. Indeed, there is now growing evidence to suggest that changes in muscle size and strength are not as closely associated as one might think given the strong relationship between the two variables at baseline. In their study, Erskie et al. (2014) reported that increases in muscle size accounted for 19% of the improvement in strength following resistance exercise. However, in line with the present study, the majority of the literature report changes in muscle size accounting for approximately 3-5% of the change in muscle strength (Balshaw, Massey et al. 2017, Buckner, Dankel et al. 2016). Instead, it appears that neuromuscular adaptations explain a much larger proportion of the improvements in strength (Balshaw, Massey et al. 2017). Whilst there is some evidence for improvements in neuromuscular function following exercise in CKD populations (Molsted, Andersen et

al. 2013), more research is needed investigating neuromuscular adaptations to exercise intervention in non-dialysis CKD.

5.7.3 Muscle hypertrophy following exercise

In addition to improvements in muscle strength, performing both 12-weeks of regular AE or CE resulted in increase quadriceps muscle volume measured by MRI. In the current study AE performed thrice weekly for 30 minutes at an intensity of 70-80% of VO_{2peak} resulted in a 4.3% increase in total quadriceps measured volume using MRI, whilst in the CE group, the addition of progressive resistance exercise performed on two of the three sessions at 70% of the individuals estimated 1-RM resulted in 9.4% increase in quadriceps volume. Again, these data suggests that CE results a greater degree of hypertrophy, being double that of those performing AE.

There is good evidence that progressive resistance training performed on its own results in muscle hypertrophy in CKD populations (Cheema, Chan et al. 2014, Watson, Greening et al. 2014). However, the effects of combining progressive resistance training with aerobic exercise on muscle hypertrophy in non-dialysis CKD has not previously been investigated. Moreover, evidence from previous work by our group suggested that aerobic exercise may result in abnormal amino acid metabolism within the muscle of CKD patients resulting in a less favourable anabolic environment, and thus preventing any increase in muscle size from anabolic stimuli (i.e. resistance exercise). However, the increases in RF-ACSA of 8.8% and quadriceps volume of 9.4% reported following CE in the present study are similar to that seen in previous work by our group (Watson, Greening et al. 2014) where increases of 8.3% and 9.8% increase in CSA and volume, respectively were reported following an 8-week PRT intervention performed 3x/week at the same training load (70% 1-RM) in individuals with CKD stages 3b-4.

The present study also provides evidence for the role of aerobic exercise in improving muscle mass of the lower limbs in non-dialysis CKD. Whilst aerobic exercise has a number of benefits, it is not typically thought of as anabolic, and current evidence for this

within CKD populations is equivocal. In a similar 12-week aerobic exercise intervention performed 3x/week, Baria et al (2014) reported an increase in leg lean mass measured by DXA in obese CKD patients. In contrast, a 6-month aerobic exercise intervention of home-based walking 5x/week reported no change in lean mass (Kosmadakis, John et al. 2012). Whilst both studies used DXA to measure lean body mass, it is likely that the methodology (i.e. total vs. segmental) may have led to the discrepancy between studies. In the present study quadriceps muscle hypertrophy was observed using ultrasound and the gold standard MRI, which allows for the direct measurement of muscle size in a specific region of interest.

It is plausible that when performed at a sufficient intensity, duration and frequency, aerobic exercise produces a large number of muscle contractions, placing a high-volume, low-load stimulus on exercising muscles. This stimulus may alter the molecular regulation and protein metabolism that is conducive to increased myofibre and whole muscle size, particularly in sedentary populations (Konopka, Harber 2014). Indeed, a series of studies conducted in haemodialysis patients, reported a significant reduction in myostatin mRNA and a concurrent increase in growth factors IGF-1 and its receptor, favouring an anabolic environment following endurance exercise training (Kopple, Wang et al. 2007). Subsequent chapters of this thesis investigate the molecular response to AE and CE in this cohort.

Taken together, these data suggest that both AE alone and CE can result in increases in muscle mass and strength in non-dialysis CKD. However, combined aerobic and resistance exercise confers superior improvements in both muscle mass and strength above that of AE alone.

5.7.4 Physical performance

Positive mean changes in assessments of physical performance occured following exercise. Both groups exhibited mean changes indicative of improvements in STS-5 and -60 scores as surrogate measures of lower extremity muscle power and endurance,

respectively. The changes of 15% for the STS-5 and 35% for STS-60 following AE tended to be greater than performing CE with changes of 9% and 21% for STS-5 and STS-60, respectively. There was however, a considerable amount of variation within the individual response to these tests, which is most likely reflected by the difference in p values, despite the larger mean change occurring in the aerobic group (Table 5.3). Furthermore, it is important to note that the changes observed for the STS-5 following exercise are lower than observed following the 6-week control period. Given the lower reliability of this test reported earlier, it is difficult to draw conclusions from the data in the present study.

Both groups showed similar mean improvements in ISWT performance of 6% and 8% following AE and CE, respectively. The ISWT provides a measure of patients walking ability and a surrogate of exercise capacity, indicating improvements in these parameters following 12-weeks supervised exercise. Improvements in functional capacity following exercise training have been reported in a number of systematic reviews that include non-dialysis CKD. In line with the present results Greenwood et al (2012) reported improvements in STS-60 of 26% following a 12-week renal rehabilitation programme consisting of combined aerobic and resistance exercise, however the authors report greater improvements in ISWT performance with a 60% increase in distance walked. The greater increase in ISWT seen by Greenwood and colleagues (2012) may be attributed to participants being more deconditioned at baseline, with mean distance walked on the ISWT being 200m in comparison to 418m observed in the present cohort of non-dialysis participants prior to the commencement of exercise.

Whilst the ISWT provides a surrogate measure of exercise capacity, only modest mean changes in absolute and relative VO_{2peak}, the 'gold standard' measure of cardiorespiratory fitness and exercise capacity were observed following both interventions. Two systematic reviews and meta-analyses investigating the effects of exercise in CKD populations state that any type of exercise will significantly improve aerobic capacity in CKD stages 2-5 (Heiwe, Jacobson 2011, Heiwe, Jacobson 2014). However, whilst the mean changes of 1.07 ml/kg/min (5%) following AE and .63 ml/kg/min (3%) following CE did not reach statistical significance, they are similar to the pooled mean changes of 0.56-0.82

ml/kg/min reported in the aforementioned meta-analyses (Heiwe, Jacobson 2011, Heiwe, Jacobson 2014). Given that the combined sample sizes of the meta-analyses range from 847-928 participants, it is likely that the current study is not sufficiently powered to detect such a difference.

It is possible that interventions of a longer duration are required to elicit greater improvements in VO_{2peak}. Indeed, Headley et al. (2012) reported an 8% increase in VO_{2peak} following 48-weeks of predominantly aerobic exercise in non-dialysis CKD, whilst Greenwood et al. (2015) reported an improvement of 14% in VO_{2peak} following 12-months of regular exercise, but not at 6-months, supporting the notion that longer durations may be required to elicit substantial improvements in VO_{2peak}. Interestingly, in addition to the modest changes in aerobic capacity, similar mean increases in peak wattage achieved during the exercise test were observed following both exercise interventions. The increase in peak wattage may indicate improvements in exercise tolerance, however these could not attributed to the increases in lower limb muscle mass or strength observed.

Taken together, modest changes in physical performance parameters were observed that are indicative of positive effects following both AE and CE. Whilst the study was underpowered to detect statistical differences between groups, mean changes observed were in line with those previously reported following exercise interventions in non-dialysis CKD. However, the lack of statistical power prevents any conclusions being drawn regarding the superiority of one mode of exercise for improving physical performance in non-dialysis CKD.

5.8 Limitations

5.8.1 Statistical power

As the primary purpose of the ExTra CKD study was to generate biological samples to extend previous work by our group, the sample size was not powered to detect statistical differences in the outcomes reported above. Whilst we report mean changes for the outcome measures following exercise, it is likely that the study was underpowered to detect within group changes (i.e. following AE or CE) or between group differences (i.e. AE vs. CE), therefore the changes reported above should be confirmed in appropriately designed randomised trials with sufficient statistical power. However, this data helps to describe the effects of different modes of exercise training in non-dialysis CKD on skeletal muscle mass and function, and should provide useful information to help inform future exercise guidelines in this population.

5.8.2 Participant dropout

There was a high participant dropout (33%) over the course of the entire trial. However, it should be noted that the majority of participant dropout (22%) occurred prior to randomisation, during the non-exercise control period (Figure 5.1), and prior to exercise initiation. The participants that dropped out during the exercise phase of the trial were not treatment (exercise) related and therefore excluding them from the analysis did not bias the results. This was confirmed by the Little's MCAR test performed on the data and the sensitivity analysis.

5.8.3 Baseline differences between groups

Whilst the groups appreaerd to be well matched for age and eGFR at baseline, a greater number of participants in the AE group had comorbid conditions, with the median blood pressures and serum cholesterol being higher in this group also. Although an attempt was made to control for these differences between groups within the statistical analysis (e.g. accounting for the presence of diabetes) it is likely that participants in the AE group would

have been reciving a greater number and/or dosages of medications that may have confounded the results of the present analyses.

5.8.4 Blinding of assessors and participants

Due to the nature of the study it was not possible to blind participants and assessors to the exercise group allocation. However, a researcher independent from the study protocol randomised participants. The same researchers that supervised the exercise sessions were also responsible for conducting outcome assessments due to limited resources. To prevent bias, the same instructions and encouragement where appropriate were provided for every participant. Finally, as stated in the methods, one assessor (DG) performed all imaging analysis, however, a researcher separate to the study protocol anonymised all MRI images to avoid bias in image analysis.

5.8.5 Exercise volume

In an attempt to match groups for the total volume of exercise undertaken over the 12-week period, the duration of AE was reduced on the two sessions per week that the CE group performed resistance exercise. This resulted in the AE group performing an additional 20 minutes of aerobic exercise per week. This equated to an additional 240 minutes or 8 sessions (~3 weeks training 3/x week) of 30 minutes aerobic exercise. This may be reflected in the slightly greater mean changes in VO_{2peak} seen in the AE group. It may also suggest that 70 minutes of aerobic training (as done by the CE group), over the 90 minutes (as done by the AE group) is enough to elicit positive changes in physical performance. Further, the addition of 20 minutes of weekly RT seemingly has comparable benefits (in regard to physical function) and superior benefits (in terms of muscle mass) than doing an additional 20 minutes of aerobic exercise.

5.9 Conclusions

In patients with non-dialysis CKD both supervised AE and CE resulted in increases in quadriceps muscle strength and size. However, these improvements were greater in those performing CE, showing increases that were double that of the AE only group. Both groups also showed similar mean changes with no statistical differences in physical performance parameters indicative of a positive effect of exercise on functional parameters and exercise capacity. However, the study was not powered to detect within or between group differences in these measures and further large-scale appropriately designed trials are needed to investigate these further. Overall, these observations suggest that combined aerobic and progressive resistance exercise result in greater improvements in muscle size and strength compared to aerobic exercise performed alone.

Chapter 6

Skeletal Muscle Molecular Responses to Aerobic or Combined Aerobic and Resistance Exercise Training in non-dialysis CKD

6.1 Introduction

Chronic kidney disease is a catabolic condition that results in the loss of protein stores termed PEW (Obi, Qader et al. 2015). Skeletal muscle wasting is a hallmark of protein energy wasting, resulting from the loss of muscle proteins (Obi, Qader et al. 2015). Although PEW is most commonly reported in ESRD patients receiving haemodialysis, skeletal muscle wasting and dysfunction are prevalent amongst patients with earlier stage CKD. As discussed in previous chapters, this loss of muscle is associated with poor physical performance (Segura-Orti, Gordon et al. 2017), increased risk of mortality (Pereira, Cordeiro et al. 2015), and cardiovascular events (Harada, Suzuki et al. 2017), independent of kidney function (see section 2.3.3). Where many of the underlying causes of renal impairment are largely irreversible, importantly, skeletal muscle wasting and dysfunction may be reversible features of the disease process. This is demonstrated by the efficacy of appropriate exercise training interventions that are capable of increasing muscle mass and function in CKD populations, as demonstrated in Chapter 5 of this thesis.

The aetiology of muscle wasting and dysfunction in CKD is complex and has numerous contributing factors, such as uraemia, inflammation, insulin resistance, malnutrition, metabolic acidosis, hypermetabolism, and physical inactivity (Stenvinkel, Lindholm et al. 2004, Wang, Mitch 2014) which ultimately result in alterations to muscle protein metabolism. CKD induces muscle protein degradation through the activation of caspase-3 and the UPS (Du, Wang et al. 2004, Bailey, Wang et al. 1996, Rajan, Mitch 2008), whilst concurrently suppressing synthesis of new proteins. However, these processes are not separate and appear to converge on the insulin/IGF-1 signalling pathway, which plays a pivotal role in the muscle wasting processes in CKD (Zhang, Wang et al. 2010, Bailey, Zheng et al. 2006).

Central to this process is the phosphorylation of Akt (p-Akt), which plays an important role in the maintenance of muscle mass, and forms a crossroads between protein degradation and synthesis (Bodine, Stitt et al. 2001). P-Akt results in the phosphorylation of FOXO1 and FOXO3a transcription factors leading to their exclusion from the nucleus,

and therefore reduced protein degradation via the UPS (Glass 2010). At the same time, p-Akt also leads to phosphorylation of downstream targets, mTORC1 and P70S6K, which mediate protein synthesis (described in section 2.6.1). However, evidence from animal models of CKD suggests that the muscle wasting observed in this patient population occurs with decreased p-Akt resulting in increased production of muscle specific ubiquitin E3 ligases MuRF1 and MAFbx (Stitt, Drujan et al. 2004, Sacheck, Ohtsuka et al. 2004) and subsequently UPS activation (Lecker, Goldberg et al. 2006). Consequently, the expression of MuRF1 and MAFbx is frequently used as a marker of muscle protein degradation. In order for proteolysis to occur via the UPS, actomyosin proteins must first be cleaved into fragments by caspase-3 resulting in a 14-kDa actin fragment, which again has been proposed as a marker of muscle catabolism (Workeneh, Rondon-Berrios et al. 2006).

In the general population exercise has a profound effect upon skeletal muscle metabolism, and elicits a number of phenotypical adaptations that are beneficial for health. Indeed, there is now growing evidence that exercise is an intervention that has a multitude of benefits in CKD patients, including improved muscle mass, physical functioning, and exercise capacity (Cheema, Chan et al. 2014, Heiwe, Jacobson 2011, Heiwe, Jacobson 2014). However, only a few studies have investigated how exercise affects the mechanisms that contribute to muscle wasting and dysfunction in CKD. In animal models of CKD, a series of studies have shown that muscle overloading as a model of resistance exercise increases the gene expression of IGF-1 in addition to increased phosphorylation of downstream mediators of IGF-1 signalling pathway (Sun, Chen et al. 2006, Chen, Sood et al. 2008). Similarly, Wang et al (2009) used plantaris muscle overloading and treadmill running to investigate the effect of different modes of exercise on muscle protein metabolism in CKD. They reported that overload was effective at reducing proteolysis and increasing intracellular signals of IGF-1 signalling pathways that regulates protein synthesis, ultimately resulting in increased muscle mass. In contrast, treadmill running was effective at suppressing muscle proteolysis but it did not reverse the CKD-induced suppression of protein synthesis.

There is some evidence of similar effects in humans with CKD. In patients with ESRD receiving haemodialysis, it has been shown that a single bout of resistance exercise can augment muscle protein anabolism (Majchrzak, Pupim et al. 2008), whilst regular exercise in haemodialysis patients may induce changes in skeletal muscle gene expression which are conducive to muscle anabolism. Indeed, Kopple et al (2007) investigated the effects of performing regular aerobic, resistance, or a combination of both modes in haemodialysis patients reporting increases in IGF-1 gene expression following regular exercise, in addition to a reduction of myostatin mRNA expression, indicative of changes favoring muscle anabolism. Furthermore, in the same study the authors reported reductions in the 14-kDa actin fragments in biopsies from patients performing aerobic exercise only or in combination with resistance exercise, indicative of reduced protein catabolism via the caspase-3 system. However, despite these changes favoring muscle anabolism, no changes in muscle mass were reported.

Further to impairments in the above-mentioned signalling pathway, an altered amino acid metabolism also contributes to muscle wasting in CKD patients. It is well established that the BCAAs, in particular leucine are capable of stimulating muscle protein synthesis, through direct activation of the mTORC1 signalling pathway (Moberg, Apro et al. 2016, Rennie, Bohe et al. 2006). In an early study it was identified that haemodialysis patients exhibit depletion of free intramuscular amino acids, which is thought to be one of the initial steps in the wasting process (Bergstrom, Alvestrand et al. 1990). The depletion of intramuscular amino acids appears to be partly a result of metabolic acidosis, and is reversed by alkali therapy (Lofberg, Wernerman et al. 1997). Furthermore, evidence from *in vitro* models suggests that CKD induced acidosis affects amino acid transport into the muscle cell (Evans, Nasim et al. 2007) resulting in proteolysis (Evans, Nasim et al. 2008). Both depletion of free amino acids and impaired availability are likely to contribute to reduced muscle protein synthesis in CKD (Adey, Kumar et al. 2000, Nair 2005).

Whilst resistance exercise may be beneficial at improving muscle protein metabolism in CKD through increasing protein synthesis, the effects of aerobic exercise on muscle protein metabolism in CKD is equivocal. Whilst muscle hypertrophy following aerobic exercise was observed in Chapter 5, improvements in muscle mass in other studies are

equivocal (Kopple, Wang et al. 2007, Kosmadakis, John et al. 2012). One proposed explanation for this is that H^+ ions generated through aerobic exercise may result in a transient worsening of acidosis and therefore affect muscle protein metabolism and amino acid concentrations (Clapp, Bevington 2011). Indeed, previous work from our group investigated the effects of 6 months walking exercise in patients with CKD stage 4-5 who were randomised to one of four groups; control, control + additional bicarbonate, exercise, exercise + additional bicarbonate in order determine the effects of exercise on acidosis, and skeletal muscle. Exercising patients taking additional bicarbonate was accompanied by a significant reduction of MuRF1 mRNA indicating reduced catabolism, however indicators of muscle anabolism remained unchanged and no improvements in muscle mass were seen (Watson, Kosmadakis et al. 2013, Kosmadakis, John et al. 2012). Furthermore, the authors observed a depletion of intramuscular free amino acids including the BCAA leucine in the exercise only group; this was corrected in those receiving additional bicarbonate (Watson, Kosmadakis et al. 2013). This may have important implications for patients when performing aerobic exercise and may compromise the ability to increase muscle mass when combining aerobic and resistance exercise which is traditionally more anabolic and capable of increasing muscle mass in CKD populations (Cheema, Chan et al. 2014, Watson, Greening et al. 2014).

When taking the previous work from our group together with the data from the above mentioned studies in animal models and haemodialysis patients, it implies that aerobic exercise may be beneficial at suppressing muscle protein degradation in CKD, but may not provide sufficient anabolic stimulus to restore abnormalities in protein synthesis. Furthermore the depletion of intramuscular free amino acids seen in patients performing aerobic exercise without additional bicarbonate identifies the need for further research to determine if this may impair the ability to increase muscle mass when combined with other anabolic therapies (i.e. resistance exercise).

6.2 Aims and hypotheses

In view of the above, this chapter aimed to investigate the molecular responses to aerobic and combined aerobic and resistance exercise by addressing the following hypotheses:

1. CE would elicit greater increases in factors associated with muscle protein synthesis (Akt, mTORC1, and P70S6K)

2. Both CE and AE would suppress factors associated with degradation (MuRF1, MAFbx, and 14-kDa actin fragment)

6.3 Methods

A detailed description of all methods utilised in this chapter are provided in the general methods section (Chapter 3). For plasma and muscle amino acid analyses that only appear in this chapter, a full description of methods is provided.

6.3.1 Study design and participants

Participants included in this chapter are a sub group of those who successfully completed the ExTra CKD trial (ISRCTN registry registration number: 36489137) described fully in Chapter 3.

6.3.2 Exercise interventions

Exercise interventions have been described fully in Chapter 3.

6.3.3 Muscle biopsy sampling and handling

Biopsies were taken from the middle portion of the vastus lateralis muscle of the individuals' right leg using a 12-gauge microbiopsy needle and were obtained on 3 occasions following an overnight fast. Biopsies were obtained at baseline (B1), and then 24h after the first (B2), and final (B3) exercise sessions, to investigate the acute response to exercise in the untrained and trained states, respectively. Samples were immediately snap-frozen and stored in liquid nitrogen until analysis.

6.3.4 Blood sampling

Fasted venous blood samples were collected at the same time as the muscle biopsies into S-Monovettes® (Sarstedt Ltd., Leicester, UK) containing K2 EDTA-Gel anticoagulant, and the plasma stored at -80°C for later analysis of amino acid concentrations.

6.3.5 Measurement of intramuscular and plasma amino acids by high performance liquid chromatography (HPLC)

6.3.5.1 Preparation of muscle biopsies for amino acid analysis

Approximately 10-15mg/ww of muscle was homogenised (MP FastPrep-24® 5G, MP Biomedicals) in 40μl/mg of 0.3M PCA containing 1mM Norvaline as an internal control, transferred to a fresh tube and incubated on ice for 30 minutes. Samples were then centrifuged at 13,000rpm for 15 minutes at 4°C and the supernatant removed to a fresh tube and placed on ice. Following this, an equal amount of freon-trioctylamine (78% v/v 1,1,2-trichlorotriluoroethane, 22% v/v trioctylamine) was added to the supernatant and vortexed for 1 minute. The tubes were then microcentrifuged briefly (~10 seconds) at 13,000rpm to separate the sample into three phases. Of the three phases, approximately 80% of the top aqueous phase was taken and filtered through a 0.45 microfilter into an Eppendorf tube and stored at -80°C until analysis.

6.3.5.2 Preparation of plasma samples for amino acid analysis

Corresponding plasma samples collected at the same time as muscle biopsy samples were used to determine plasma amino acid concentrations. 12.5μl of 12M (70%) PCA containing 40mM Norvaline (internal standard) was added to 500μl of plasma and vortexed for 1 minute before being incubated on ice for 30 minutes. From this point, amino acid extraction was performed using Freon - following the method described above for muscle biopsies.

6.3.5.3 Amino acid separation by reverse-phase HPLC

Amino acid concentrations were determined by reverse phase HPLC. A fresh amino acid master standard was used each time samples were run, containing 25μl Agilent amino acid standard (5061-3330), 75μl nano pure water and 1μl supplementary amino acid mixture (amino acid concentrations of Agilent and supplementary standards can be found in Appendix B). All samples were separated on Agilent (1100 Series) chromatograph with ultraviolet (UV) detection using a Zorbax eclipse AAA column (4.6 x 75mm, 3.5μm) with pre-column derivitisation using Orthophalaldehyde (OPA; Agilent, UK, 5061-3335) and Fluorenylmethyloxycarbonyl chloride (FMOC; Agilent, UK, 5061-3337). Each sample was eluted with 40mM sodium dihydrogen orthophosphate solution pH 7.8 supplemented with a linear gradient of 0-57% v/v acetonitrile:methanol:water (45:45:10) applied between minutes 1 to 9.8 in each run. At the end of each run the column was purged with 100% acetonitrile:methanol:water for 2 minutes and then re-equilibrated with 100% sodium dihydrogen solution orthophosphate pH 7.8 (Appendix A) for a further 2 minutes, before moving onto the next sample. Analysis of all biopsy and plasma samples from the same patient was performed at the same time. Peaks were integrated using the Agilent Chemstation software and amino acid concentrations calculated in relation to the standard.

6.3.5.4 Determination of chloride concentration of muscle and plasma samples

Extracellular water content was calculated from the chloride concentrations of muscle and plasma samples determined by turbidimetry of neutralised muscle and plasma samples obtained through the methods described above. Plasma samples were diluted

1:100 using ultra pure water prior to performing the assay. Following this, 50µl of chloride standard (0 – 1mM) or sample was added to 50µl of Precipitating Reagent (5mM Silver Perchlorate, 4% dextran in 2M PCA) into a 1.5ml Eppendorf tube and vortexed. Subsequently, 95µl was transferred into corresponding wells of a 96 well plate and incubated for 2h at ambient temperature, before reading at 405nM on a Multiskan™ FC spectrophotometer (Thermo, Scientific, UK). The contribution of trapped extracellular amino acids in the biopsies was subtracted from the total biopsy amino acid content, assuming that concentrations in trapped extracellular water equalled the concentrations measured per litre of plasma water.

6.3.6 Determination of phosphorylated proteins and 14-kDa actin fragment content of muscle biopsies by Western blotting

Western blotting was used to determine the phosphorylation status of Akt (Ser^{473}), mTOR (Ser^{2448}), and P70S6K (Thr^{389}) in the muscle biopsies of participants. This technique was also used to determine the protein content of 14-kDa actin fragment which gives an indication of caspase-3 activity (Workeneh, Rondon-Berrios et al. 2006). For phosphorylated proteins GAPDH was used as a loading control, whilst the 14-kDa actin fragment was normalised to 42-kDa Actin content. A detailed description of the western blot procedure can be found in Chapter 3.

6.3.7 Determination of MuRF-1 and MAFbx mRNA expression by quantitative RT-PCR

The mRNA expression of the ubiquitin E3 ligases MuRF1 and MAFbx were determined using qRT-PCR following the procedures for RNA extraction and PCR experimentation outlined in Chapter 3. The Ct values from each of the genes of interest were normalised to the Ct values of 18S as the reference gene, and relative changes in expression (fold changes) were calculated using the $2^{-\Delta\Delta Ct}$ method which sets the baseline value to 1.0. Primers and probes for all genes were supplied as Taqman gene expression assays

(Applied Biosystems, Warrington, UK, MuRF1 Hs00261590_m1, MAFbx Hs01041408_m1, and 18s Hs99999901_s1).

6.3.8 Statistical analysis

A full description of the statistical analysis performed for laboratory variables can be found in section 3.17. Briefly, the distribution of the data was assessed using the Shapiro-Wilks test. Variables that were not normally distributed were log-transformed for analysis, and back transformed for presentation. A primary analysis was performed for each variable using repeated measures ANOVA for biopsy time-point (baseline, 'untrained', and 'trained') and group allocation (AE and CE), and the partial eta squared (ηp^2) effect size statistic calaculated (interpreted as: 0.01 small, 0.06 medium, and 0.14 large effect sizes)

In addition, paired t-tests were performed between specific time points (baseline - untrained, baseline - trained, and untrained - trained) following AE and CE and expressed as mean change (90% CIs). Magnitude based inferences were used to estimate the mean effects between AE and CE groups. The percentage chances of the effect being substantially positive (increased), trivial or substantially negative (decreased) were assessed and qualitative inferences assigned to the quantitative percentile scores as follows: 25-75% *possible*, 75-95% *likely*, and >99% *most likely* (Batterham, Hopkins 2005). Effects were deemed unclear if there was a greater than 5% probability of being both substantially positive and substantially negative (Batterham, Hopkins 2005). Linear mixed models and within group comparisons were carried out using IBM SPSS Statistics (IBM, Chicago, IL) Version 24, whilst effect sizes and MBI were calculated using custom Microsoft Excel spread sheets (Hopkins 2007). Statistical significance was accepted at p <0.05.

6.4 Results

6.4.1 Participant demographics

Of the 36 participants that completed the ExTraCKD study described in the previous chapter (Chapter 5), 19 (AE n=10 and CE n=9) consented to give multiple skeletal muscle biopsies. Patient demographics can be found in Table 6.1. Data from all 19 participants were included in the RT-PCR analysis of MuRF1 and MAFbx gene expression. However, two patients were excluded from western blotting analysis as the biopsy was not fasted (AE n=1) or there was not sufficient biopsy material (n=1). As such results from western blotting experiments described below are from 17 patients (AE n=9 and CE n=8).

6.4.2 Intramuscular BCAA concentrations

No significant interactions were observed for any of the individual intramuscular BCAAs or total intramuscular BCAA concentrations. MBI analysis adjusted for baseline values revealed that the change in intramuscular valine following CE accustomed exercise in the trained stated was *likely* reduced (94% likelihood of at least a small effect) compared to those performing AE. All other between group comparisons following unaccustomed and accustomed exercise were deemed *unclear* (Table 6.2). The concentrations of all intramuscular amino acids measured in response to exercise can be found in Appendix B.

6.4.3 Plasma BCAA concentrations and amino acid concentration
gradients

No time or group x time interactions were observed on plasma BCAA concentrations. Changes in fasted BCAA concentrations following AE and CE in the untrained and trained state can be found in Table 6.3. Between group comparisons were all deemed *unclear* according to the MBI analysis. The concentrations of all plasma amino acids

measured in response to exercise can be found in Appendix B. No effects of either AE or CE were obsereved on amino acid concentration gradients (data not shown).

Table 6.1 Participant demographics

	Aerobic	Combined
Number of patients	10	9
Age (years)	65 ± 8	59 ± 18
Sex (*n* men/women) (% women)	4/6 (60%)	3/6 (50%)
eGFR (mL/min/1.73m^2)	27 ± 9	27 ± 6
Serum bicarbonate (mmol/L)	25 ± 6	26 ± 5
Height (cm)	169 ± 10	168 ± 10
Weight (kg)	84 ± 14	84 ± 15
BMI (kg/m^2)	30 ± 6	30 ± 5
ALMI (kg/m^2)	7.42 ± 1.12	7.86 ± 0.90
RF-CSA (cm^2)	9.9 ± 3.3	9.2 ± 1.9
***RF-CSA increase (%)**	2%	7%
Quadriceps Vol. (cm^3)	1032.5 ± 299.9	1085.4 ± 275.2
***Quadriceps Vol. increase (%)**	3%	10%
Diabetes (*n*)	1	1

Data presented as mean $\pm$ SD. Abbreviations: eGFR = estimated glomerular filtration rate; BMI = body mass index; ALMI = appendicular lean mass index; RF-CSA = rectus femoris cross-sectional area; Quadriceps vol. = quadriceps volume. *% change following 12-weeks exercise

Table 6.2 Change in intramuscular BCAA concentrations following aerobic and combined exercise

Amino Acid	Time point	Aerobic		Combined		AE vs. CE		
		Mean %Change (90%CI)	*p*	Mean %Change (90%CI)	*p*	%Difference in CE (90% CI)	*d* (90% CI)	Inference
L-Leu	B1-B2	+5 (-22 to 40)	.766	-14 (-59 to 80)	.104	-19 (-70 to 17)	-.56 (-1.59 to .46)	*Unclear*
	B1-B3	+4 (-25 to 40)	.829	+9 (-47 to 120)	.454	+10 (-26 to 64)	.23 (-.79 to 1.25)	*Unclear*
	B2-B3	-1 (0.05 to 0.58)	.939	+26 (40 to 390)	.008	+34 (3 to 74)	.72 (0.07 to 1.38)	*Likely* ↑ (91%)
L-Val	B1-B2	-2 (-29 to 40)	.909	-5 (-70 to 30)	.760	-20 (-55 to 43)	-.32 (-1.14 to .51)	*Unclear*
	B1-B3	+40 (0 to 90)	.087	-11 (-31 to 20)	.412	-43 (-67 to -2)	-.75 (-1.47 to -.03)	*Likely* ↓ (94%)
	B2-B3	+44 (-3 to 110)	.120	-6 (-25 to 20)	.621	-29 (-54 to 9)	-.66 (-1.49 to .17)	*Unclear*
L-Ile	B1-B2	-25 (-64 to 60)	.485	-26 (-63 to 50)	.363	+2 (-54 to 124)	.05 (-2.13 to 2.22)	*Unclear*
	B1-B3	-32 (-68 to 40)	.357	+183 (-71 to 270)	.406	+36 (-68 to 476)	.41 (-1.52 to 2.34)	*Unclear*
	B2-B3	-9 (-48 to 60)	.753	+285 (-64 to 560)	.367	+84 (-90 to 397)	.82 (-3.05 to 4.69)	*Unclear*
Total BCAA	B1-B2	-10 (-22 to 100)	.213	-5 (-23 to 40)	.789	+1 (-31 to 48)	.02 (-.90 to .94)	*Unclear*
	B1-B3	+31 (-20 to 110)	.330	+3 (-18 to 50)	.880	-20 (-51 to 31)	-.45 (-1.45 to .55)	*Unclear*
	B2-B3	+46 (-16 to 150)	.233	+8 (-14 to 40)	.536	-20 (-48 to 22)	-.46 (-1.32 to .40)	*Unclear*

Key: Time point = biopsy time point where; B1-B2 = change in concentration from baseline in response to acute exercise in the untrained state, B1-B3 = change in concentration from baseline in response to acute exercise in the trained state, B2-B3 = difference between response in untrained and trained states; Inference = qualitative inference (probability of at least a small effect %); L-Leu = Leucine; L-Val = Valine, L-Ile = Isoleucine; Total BCAA = sum of branched chain amino acids. *d* = effect size where; 0.2 = small, 0.5 = moderate, and >0.8 = large effect.

Table 6.3 Change in plasma BCAA concentrations following aerobic and combined exercise

Amino Acid	Time point	Aerobic Mean %Change (90%CI)	p	Combined Mean %Change (90%CI)	p	AE vs. CE %Difference in CE (90% CI)	d (90% CI)	Inference
L-Leu	B1-B2	+1 (-31 to 50)	.766	-6 (-13 to 10)	.179	-4 (-18 to 14)	-.10 (-.56 to .36)	*Unclear*
	B1-B3	+3 (-12 to 20)	.730	-4 (-26 to 30)	.775	-7 (-28 to 21)	-.18 (-.84 to .47)	*Unclear*
	B2-B3	+2 (-9 to 10)	.753	+3 (-17 to 30)	.797	-4 (25 to 24)	-.01 (-.68 to .60)	*Unclear*
L-Val	B1-B2	-1 (-17 to 20)	.919	-8 (-16 to 0)	.119	-4 (-18 to 13)	-.11 (-.55 to .34)	*Unclear*
	B1-B3	-3 (-12 to 10)	.580	-7 (-28 to 20)	.594	-2 (-23 to 26)	-.05 (-.73 to .64)	*Unclear*
	B2-B3	-2 (-15 to 10)	.796	+1 (-29 to 40)	.958	+2 (-22 to 34)	.06 (-.64 to .75)	*Unclear*
L-Ile	B1-B2	+9 (-12 to 40)	.473	-6 (-17 to 10)	.371	-8 (-23 to 9.5)	-.18 (-.55 to .19)	*Unclear*
	B1-B3	+6 (-11 to 30)	.536	+8 (-30 to 70)	.740	+13 (-16 to 35)	.25 (-.28 to .77)	*Unclear*
	B2-B3	-3 (-15 to 10)	.673	+25 (-29 to 20)	.459	+4 (-21 to 37)	.07 (-.44 to .58)	*Unclear*
Total BCAA	B1-B2	+1 (-19 to 30)	.935	-4 (-9 to 0)	.220	-2 (-16 to 15)	-.05 (-.58 to .47)	*Unclear*
	B1-B3	0 (-90 to 130)	.997	+1 (-8 to 10)	.846	-5 (-26 to 22)	-.16 (-.88 to .57)	*Unclear*
	B2-B3	+1 (-11 to 20)	.890	+5 (-11 to 20)	.581	+4 (-22 to 30)	.10 (-.68 to .89)	*Unclear*

Key: Time point = biopsy time point where; B1-B2 = change in concentration from baseline in response to acute exercise in the untrained state, B1-B3 = change in concentration from baseline in response to acute exercise in the trained state, B2-B3 = difference between response in untrained and trained states. Inference = qualitative inference (probability of at least a small effect %); *M. Likely* = most likely; *V. Likely* = very likely. L-Leu = Leucine; L-Val = Valine, L-Ile = Isoleucine; Total BCAA = sum of branched chain amino acids. *d* = effect size where; 0.2 = small, 0.5 = moderate, and >0.8 = large effect

6.5 Effects of exercise on phosphorylated proteins

6.5.1 Phosphorylation of Akt^{ser473}

P-Akt^{ser473} exhibited a significant interaction and large effect for biopsy time point (F=3.613, ηp^2=.194, p=.016) and large effect for group x time (F=2.553, ηp^2=.145, p=.095). Following unaccustomed exercise, the effect of CE in comparison to AE was deemed *unclear* with both AE and CE demonstrating mean reductions in p-Akt (Table 6.4). Following accustomed exercise, a mean reduction of -22% (-43 to 40) in p-Akt was observed in the AE group, whilst CE resulted in a mean increase in p-Akt of +118% (30 to 280), which was deemed *likely* (79% of at least a small effect) greater than that of the AE group when accounting for baseline values (Table 6.4). The p-Akt response to unaccustomed and accustomed AE and CE is shown in Figure 6.1.

6.5.2 Phosphorylated P70S6K^{Thr389}

The phosphorylation of P70S6K1 at the mTORC1 specific site Thr389 is a marker of mTOR activity (Hornberger, Sukhija et al. 2007) and was detectable in blots from 17 patients. No significant interactions and only small effects were observed in p-P70S6K^{Thr389} between time points (F=.493, ηp^2=.032, p=.615) and group x time (F=.223, ηp^2=.015, p=.801). Indeed, both AE and CE demonstrated small mean changes with wide CIs, and the between group effects were deemed *unclear* (Table 6.4).

Figure 6.1 - Percentage change in p-Akt^{ser473} in response to exercise training. (A) Representative western blot showing p-Akt^{ser473} following aerobic and combined exercise where B1 = baseline, B2 = untrained response, and B3 = trained response. (B) Mean percentage change in p-Akt^{ser473} from baseline levels (represented by a value of 0 and indicated by the horizontal line) following AE. (C) Mean percentage change in p-Akt^{ser473} from baseline levels (represented by a value of 0 and indicated by the horizontal line) following CE. (D) and (E) Individual responses in p-Akt^{ser473} corrected to GAPDH and expressed in arbitrary units (au) following AE and CE, respectively.

*Denotes $p < 0.05$ from baseline levels.

#Denoted $p < 0.05$ from untrained response

Figure 6.2 - Percentage change in p-P70S6K^{Thr389} in response to exercise. (A) Representative western blot showing p-P70S6K^{Thr389} following AE and CE where B1 = baseline, B2 = untrained response, and B3 = trained response. (B) Mean percentage change in p-P70S6K^{Thr389} from baseline levels (represented by a value of 0 and indicated by the horizontal line) following AE. (C) Mean percentage change in p-P70S6K^{Thr389} from baseline levels (represented by a value of 0 and indicated by the horizontal line) following CE. (D) and (E) Individual responses in p-P70S6K^{Thr389} corrected to GAPDH and expressed in arbitrary units (au) following CE and AE, respectively.

6.5.3 Effects of exercise on protein catabolic factors

6.5.3.1 14-kDa Actin Fragment

The 14-kDa actin fragment gives an indication of caspase-3 activity, which is required for proteolysis via the UPS (Workeneh, Rondon-Berrios et al. 2006). Due to technical issues with sample preparation, 14-kDa actin content was measured in biopsies from 11 participants (AE n=6 and CE n=5). Large effect sizes and significant time (F=4.447, ηp^2=.331, p=.027) and group x time interactions (F=6.332, ηp^2=.413, p=.008) were observed for 14-kDa content. Following unaccustomed AE a mean change of +7% (-14 to 2) in 14-kDa content was obsereved in the untrained state. In comparison, unaccustomed CE resulted in a mean change of +199% (30 to 670) in 14-kDa content at the same time-point. When adjusting for baseline values, MBI analysis revealed that the change in 14-kDa content following unaccustomed CE was *very likely* (99% liklihood of at least a small effect) increased above that of the AE group (Table 6.4). In contrast, following following accustomed exercise, the between group comparison between AE and CE were deemed *unclear,* with both groups demonstrating mean reductions from baseline levels.

6.5.3.2 MuRF1 gene expression

Large and medium effect sizes were observed between time (F=2.090, ηp^2=.161, p=.230) and group x time (F=.645, ηp^2=.084, p=.539), respectively, however these did not result in significant interactions. When adjusting for baseline values, MBI analysis deemed the between groups effects to be *unclear* (Table 6.5)*.*

6.5.3.3 MAFbx gene expression

No interactions and only small effect sizes were observed between biopsy time points (F=.647, ηp^2=.021, p=.647) and group x time (F=.268, ηp^2=.016, p=.704) for MAFbx gene expression. Between group comparisons for the effects of unaccustomed and accustomed AE and CE on MAFbx were deemed *unclear* (Table 6.5).

Figure 6.3 - Change in 14-kDa content in response to exercise. (A) Representative western blot showing 14-kDa content following AE and CE where B1 = baseline, B2 = untrained response, and B3 = trained response. (B) Mean percentage change in 14-kDa content from baseline levels (represented by a value of 0 and indicated by the horizontal line) following AE. (C) Mean percentage change in 14-kDa content from baseline levels (represented by a value of 0 and indicated by the horizontal line) following CE. (D) and (E) Individual responses in 14-kDa content corrected to 42-kDa actin and expressed in arbitrary units (au) following AE and CE respectively.

Figure 6.4 - MuRF1 mRNA expression following exercise. Graphs show mean fold changes in MuRF1 mRNA expression relative to 18s following AE (A) and CE (C), and individual fold changes following AE (B) and CE (D).

#Denoted *p*<0.05 from untrained response

Figure 6.5 - MAFbx mRNA expression following exercise. Graphs show mean fold changes in MAFbx mRNA expression following CE (A) and AE (C), and individual fold changes following CE (B) and AE (D).

Table 6.4 Changes in protein phosphorylation status and 14-kDa fragment content following aerobic and combined exercise

Protein	Time point	Aerobic		Combined		AE vs. CE		
		Mean %Change (90%CI)	p	Mean %Change (90%CI)	p	%Difference in CE (90% CI)	d (90% CI)	Inference
pAkt	B1-B2	-55 (-82 to 10)	.182	-15 (-26 to 10)	.316	+27 (-61 to 318)	.18 (-.71 to 1.07)	*Unclear*
	B1-B3	-22 (-43 to 40)	.990	+118 (30 to 280)	**.030**	+89 (-17 to 333)	.50 (-.15 to 1.14)	*Likely* ⬆ (79%)
	B2-B3	+72 (0 to 170)	.080	+157 (40 to 390)	**.027**	56 (-35 to 275)	.33 (-.32 to .98)	*Unclear*
pP70S6K	B1-B2	-1 (-9 to 12)	.530	-7 (-14 to 2)	.800	-7 (-19 to 6)	-.39 (-1.1 to .31)	*Unclear*
	B1-B3	-1 (-8 to 11)	.470	-3 (-10 to 6)	.668	-3 (-13 to 9)	-.15 (-.71 to .41)	*Unclear*
	B2-B3	+1 (-11 to 15)	.561	+5 (-7 to 18)	.877	+5 (-10 to 21)	.25 (-.61 to 1.12)	*Unclear*
14-kDa Actin fragment	B1-B2	+7 (-9 to 30)	.426	+199 (30 to 670)	**.041**	+218 (47 to 586)	1.54 (.52 to 2.56)	*Very likely* ⬆ (99%)
	B1-B3	-6 (-38 to 34)	.590	-15 (-41 to 20)	.402	-18 (-60 to 65)	-.26 (-1.16 to .65)	*Unclear*
	B2-B3	-12 (-26 to 90)	.588	-72 (-113 to -9)	.082	-78 (-95 to -15)	-1.68 (-3.2 to -.18)	*Very likely* ⬇ (96%)

Key: Time point = biopsy time point where; B1-B2 = change in concentration from baseline in response to acute exercise in the untrained state, B1-B3 = change in concentration from baseline in response to acute exercise in the trained state, B2-B3 = difference between response in untrained and trained states. Inference = qualitative inference (probability of at least small effect %); *V. Likely* = very likely. pAkt = phosphorylated Akt(ser473); pP70S6K= phosphorylated P70S6K(Thr389). d = effect size where; 0.2 = small, 0.5 = moderate, and >0.8 = large effect.

Table 6.5 Changes in ubiquitin E3 ligase gene mRNA expression

Gene	Time point	Aerobic		Combined		AE vs. CE		Inference
		Fold-Change (90%CI)	*p*	Fold-Change (90%CI)	*p*	Fold-Change in CE (90% CI)	*d* (90% CI)	
MuRF1	B1-B2	2.60 (0.92 to 7.3)	.124	0.95 (0.22 to 4.1)	.950	1.06 (0.91 to 1.24)	.20 (-.29 to .69)	*Unclear*
	B1-B3	0.75 (0.46 to 1.2)	.298	0.52 (0.06 to 2.7)	.244	1.07 (0.93 to 1.24)	.25 (-.30 to .80)	*Unclear*
	B2-B3	0.29 (0.05 to 0.58)	**.030**	0.55 (40 to 390)	.544	1.00 (0.85 to 1.19)	.01 (-.59 to .62)	*Unclear*
MAFbx	B1-B2	1.29 (0.53 to 3.1)	.612	0.74 (0.23 to 2.5)	.680	1.08 (0.89 to 1.31)	.21 (-.32 to .73)	*Unclear*
	B1-B3	0.85 (0.53 to 1.4)	.541	0.66 (0.27 to 1.6)	.407	1.04 (0.93 to 1.16)	.13 (-.23 to .50)	*Unclear*
	B2-B3	0.65 (0.3 to 1.4)	.330	0.90 (0.19 to 4.3)	.902	0.97 (0.78 to 1.19)	-.10 (-.66 to .46)	*Unclear*

Key: Time point = biopsy time point where; B1-B2 = change in concentration from baseline in response to acute exercise in the untrained state, B1-B3 = change in concentration from baseline in response to acute exercise in the trained state, B2-B3 = difference between response in untrained and trained states. Inference = qualitative inference (probability of at least a small effect %), *V. Likely* = very likely. MuRF1, muscle RING finger 1; MAFbx, muscle atrophy F-box. *d* = effect size where; 0.2 = small, 0.5 = moderate, and >0.8 = large effect.

6.6 Discussion

There is growing evidence for the benefits of exercise in the non-dialysis CKD population, however very little is known regarding how exercise affects the mechanisms of muscle wasting and dysfunction to elicit physiological changes. This is the first analysis of the molecular responses to aerobic and combined exercise in patients with non-dialysis CKD at the beginning and end of a 12-week supervised exercise programme that resulted in different physiological adaptations (described in Chapter 5).

6.6.1 Intramuscular BCAA concentrations

Abnormal amino acid metabolism including, the depletion of intramuscular amino acid concentrations have previously been reported in CKD populations (Bergstrom, Alvestrand et al. 1990). To put the results of this chapter into perspective, the fasted intramuscular amino acid concentrations (see Appendix B) observed at baseline are in line with those previously reported in non-dialysis CKD patients (Watson, Kosmadakis et al. 2013). The depletion of intramuscular amino acids in CKD appears to be the result of metabolic acidosis that inhibits the transport of amino acids into the muscle cell (Evans, Nasim et al. 2007). Whilst resistance exercise is effective at increasing muscle mass in CKD, aerobic exercise may result in a transient worsening of metabolic acidosis through the generation of H^+ (Clapp, Bevington 2011). Indeed, previous work by our group observed a number of intramuscular amino acids including the BCAAs were depleted following 6-months of regular walking, something that was not observed in patients receiving additional alkali therapy (Watson, Kosmadakis et al. 2013). However, the data presented in this chapter found no evidence of such amino acid depletion following acute exercise at the start of and following completion of 12-weeks regular AE or CE (Table 6.2).

The reasons for the discrepancy between the present and previous findings could be a result of study design and biopsy sampling. This chapter aimed to investigate the acute response to exercise at the start and end of a 12-week intervention of either AE or CE (i.e.

following unaccustomed and accustomed exercise) and biopsy sampling was performed 24h following the first and last training sessions. However, in the aforementioned study by Watson et al. (2013) biopsy sampling was performed >24h following the last exercise session of a 6-month intervention and therefore reflects chronic adaptations. Other methodological differences that may have contributed to the discrepant finding include differences in the type and intensity of the exercise performed (i.e. walking vs. supervised exercise). Moreover, Watson and colleagues reported increased capacity to buffer blood lactate following exercise in those receiving additional bicarbonate, which may have contributed to the maintenance of BCAA concentrations in this group. However, owing to technical problems, no measures of blood lactate in response to AE or CE was conducted in the present study, therefore it is not possible to comment on transient worsening of acidosis following exercise. Moreover, the small number of participants in each group meant that performing sub-group analyses according to baseline serum bicarbonate level were not possible.

The maintenance of intramuscular BCAAs observed in the present chapter suggests that active amino acid transport mechanisms responsible for transport of amino acids into the muscle cell was not inhibited by acute AE or CE. However, without a measurement of the expression or activity of transporters involved in these processes such as system A (SNAT2) or system L (LAT1) (Dickinson, Rasmussen 2011), conclusions cannot be drawn regarding this. In contrast evidence exists that acute exercise, in particular resistance exercise, increases the gene expression of amino acid transporters in the skeletal muscle of healthy individuals (Dickinson, Drummond et al. 2013). Investigating the effect of AE or CE on the expression of amino acid transporters in CKD was not in the scope of the present analyses, however this merits further investigation.

6.6.2 Effects of exercise on markers of protein metabolism

6.6.2.1 Akt signalling

The insulin/IGF1-Akt signalling pathway plays a central role in the maintenance of muscle mass (Bodine, Stitt et al. 2001), with reductions in p-Akt being implicated in CKD induced muscle wasting (Bailey, Zheng et al. 2006). In animal models of CKD, 2-weeks of resistance (work overload), and to a lesser extent aerobic exercise was shown to reverse this process and partially restore signalling through Akt indicated by increased phosphorylation (Wang, Du et al. 2009). Inline with this, the present analysis observed 12-weeks of regular CE resulted in increased p-Akt by a mean of 118% (30 to 280) above baseline levels, which was deemed *likely* increased above the response of the AE group (Table 6.4). The most apparent reason for the difference in p-Akt response between CE and AE may be the greater mechanical force applied to the muscle with the addition of resistance exercise in those performing CE, which appears to be at least partly responsible for the increase in p-Akt with exercise (Sakamoto, Aschenbach et al. 2003).

Whilst p-Akt was increased following 12-weeks of CE, following unaccustomed exercise both AE and CE failed to increase p-Akt with both groups exhibiting mean reductions from baseline levels. This is in contrast to reports from the existing literature that suggest that p-Akt levels are substantially increased above baseline levels 24h following the initial bout of resistance exercise in healthy older individuals (Mayhew, Kim et al. 2009), and those with COPD (Constantin, Menon et al. 2013). Whilst the effects of resistance exercise in the present chapter may be confounded by the addition of aerobic exercise, available data investigating the molecular responses to combined aerobic and resistance exercise in healthy individuals suggests that muscle protein synthesis and associated signalling events are elevated to an equivalent level as resistance exercise only (Donges, Burd et al. 2012, Di Donato, West et al. 2014), and in some cases may enhance the response (Lundberg, Fernandez-Gonzalo et al. 2012, Fernandez-Gonzalo, Lundberg et al. 2013) suggesting no interference at the molecular level.

The lack of an increase in p-Akt following the initial bout of exercise in both groups may indicate a possible impaired response to exercise in the untrained state, and mirrors that of a previous study from our group where p-Akt was suppressed below baseline levels in the untrained state, but was substantially upregulated following 8-weeks of progressive resistance exercise (Watson, Viana et al. 2017). When taken together, the data suggests a consistent impaired response to exercise in the untrained state, the reason for which is currently unclear. However, given the important role Akt plays in the control of muscle protein metabolism, further investigation is warranted to determine potential underlying mechanisms. Furthermore, if patients exhibit a similar limited response to other anabolic stimuli such as feeding, it may provide an insight into why CKD patients experience muscle wasting. Despite this, the fact that p-Akt is improved with regular exercise incorporating resistance exercise highlights the importance of performing regular exercise incorporating resistance training in patients with non-dialysis CKD.

6.6.2.2   mTOR signalling

Downstream of p-Akt, phosphorylation of mTOR mediates protein synthesis that is increased following resistance exercise performed on its own or in combination with aerobic exercise, and remains elevated for up to 48h (Phillips, Tipton et al. 1997, Di Donato, West et al. 2014). In the present study p-mTOR^{Ser2448} was only detectable in 4 patients and therefore formal analysis was not performed. Possible explanations for this could be the fasted state of the participants when the muscle biopsy was taken, and/or the 24h time point of the biopsy. Indeed, in a study conducted in a cohort of both young and elderly individuals, Mayhew (2009) et al. reported low detection and no change in p-mTORC1 24h following unaccustomed and regular resistance exercise despite observing increases in muscle protein synthesis calculated as the fractional synthetic rate at the same time point.

In the present study, quantifiable bands were detected for p-P70S6K^{Thr389}, which is thought to be an mTOR specific phosphorylation site (Hornberger, Sukhija et al. 2007). However, no interactions were observed for biopsy time point or group allocation, and the effects of exercise between groups were deemed *unclear*. In the aforementioned

animal study, Wang et al (2009) reported overload increased protein synthesis that occurred with increased phosphorylated mTOR and P70S6K. However, no such studies have been conducted in humans with CKD.

The effects of AE on p-P70S6K are not well characterised, with the majority of the literature using resistance exercise as a model to study the pathways involved in muscle protein synthesis where p-P70S6K^{Thr389} has been shown to correlate with long-term muscle hypertrophy following resistance exercise in humans (Terzis, Georgiadis et al. 2008). Phosphorylation of P70S6K at Thr389 is considered to be an acute response phosphorylation site that is phosphorylated at 1-2h post exercise (Dreyer, Fujita et al. 2006, Kumar, Selby et al. 2009), but returns to baseline levels shortly after in the absence of feeding (Kumar, Selby et al. 2009), which most likely explains the lack of effect observed at 24h following exercise in the present study. In support of this, the aforementioned study by Mayhew and colleagues (2009) reported no detectable levels of p-P70S6K^{Thr389} at 24h following unaccustomed exercise. However, the authors did report an increase in P70S6K autoinhibitory domain phosphorylation at Thr421/Ser424 which correlated with increased myofibre cross sectional area following 16-weeks of resistance exercise. Phosphorylation of P70S6K at its autoinhibitory domain is first required for subsequent Thr389 phosphorylation prior to Thr229 phosphorylation and full kinase activity (Mayhew, Kim et al. 2009), and should therefore be investigated to gain a more complete picture of P70S6K activity in response to exercise modes in CKD.

6.6.3 Markers of muscle protein breakdown

The initial step in the breakdown of muscle proteins is the cleavage of proteins into smaller fragments by caspase-3 that are subsequently catabolised by the UPS (Du, Wang et al. 2004). This process results in an insoluble 14-kDa fragment that is detectable in muscle biopsies and provides an indication of caspase-3 activity and protein degradation (Workeneh, Rondon-Berrios et al. 2006). The present study observed differential effects of unaccustomed AE and CE on the content of the 14-kDa fragment in biopsies of CKD patients. MBI revealed that unaccustomed CE *very likely* increased 14-kDa content in comparison to AE at the same time-point. Indeed, CE resulted in a mean increase of 199%

(30 to 670) in the content of the 14-kDa actin fragment in the untrained state, however this was reduced back to below baseline levels following 12-weeks regular exercise, suggesting a possible adaptation to regular CE. Previous studies have reported reductions in 14-kDa actin fragment content in animal models following both aerobic and resistance exercise interventions (Wang, Du et al. 2009) whilst Kopple and colleagues (2007) reported reductions in biopsies of haemodialysis patients after completion of regular aerobic or combined exercise.

The data presented in this chapter is the first time the effects of acute AE or CE exercise in the untrained state and following regular exercise have been investigated, therefore differences in biopsy time points and conditions i.e. rested vs. 24h following exercise likely explain the differences between the results reported here and previous studies. Indeed, the increase in 14-kDa content of biopsies 24h following the first exercise session of CE may be a result of increased protein catabolism to clear damaged proteins. Indeed unaccustomed resistance exercise is associated with myofibrillar derangement (Gibala, MacDougall et al. 1995) and damage to sarcomeric proteins (Gibala, MacDougall et al. 1995), in addition to increased protein breakdown. Therefore it is hypothesised that increase in the 14-kDa content (indicative of caspase-3 activity) following CE in the untrained state in the present study is due to greater damage to muscle proteins as a result of the unaccustomed bout of resistance exercise, and therefore serves to remove and degrade damaged proteins, which has been associated with an increased caspase-3 gene expression in the type II fibres of healthy individuals (Yang, Jemiolo et al. 2006)

MuRF1 and MAFbx are ubiquitin E3 ligases in skeletal muscle that bind ubiquitin to substrates produced by caspase-3 tagging them for ubiquitination and subsequent degradation by the 26S proteasome. In the present study the MBI analysis revealed the effects of CE in comparison to AE were *unclear* following both unaccustomed and accustomed exercise despite exhibiting differential mean changes. The present chapter hypothesised that both AE and CE would suppress gene expression of the E3 ligases. Following unaccustomed AE, MuRF1 gene expression exhibited a mean 2.6-fold (0.92 to 7.3) increase, but was reduced below baseline levels following 12-weeks of regular exercise, suggesting a possible adaptation to regular exercise. In contrast, minimal effects

of CE were observed where modest mean reductions from baseline levels were seen in both the untrained and trained responses. Perhaps the most obvious explanation for these discrepancies is the varied individual response observed following unaccustomed CE (Figure 6.4). Indeed, 5 out of 9 participants exhibited a substantial increase in MuRF1 gene expression in the untrained state, whereas the remainder of participants exhibited a suppression below baseline levels. The reasons for such variation in the individual responses are unclear, but do not appear to be related to any participant demographics.

6.7 Limitations

It is important that the data presented within this chapter be viewed in context with its limitations. A major limitation of the data presented within this chapter is the biopsy sampling at baseline, and 24h following the first and last bouts of exercise. This limits the conclusions that can be drawn, as it is likely that a number of important signalling events will have returned to baseline levels by 24h, and therefore these data only provides a snapshot. Moreover, the lack of a second baseline biopsy before the final bout of exercise to assess the response in the trained state makes it difficult to interpret the changes observed at this time point without a true baseline biopsy being performed. Furthrmore, in the absence of direct measures of muscle protein synthesis and breakdown the results must be interpreted with caution, especially given the apparent discrepancy between phosphorylation of anabolic factors, protein synthetic rates and phenotypical adaptations (Greenhaff, Karagounis et al. 2008).

Whilst the responses to acute exercise in healthy individuals are well characterised within the literature and included in the above discussion, the lack of a healthy control group means that a direct comparison between the responses CKD and healthy individuals cannot be made, which limits the ability to make inferences specifically related to disease. Moreover, the lack of a resistance exercise only group means that conclusions cannot be drawn regarding the effects of CE in comparison to performing resistance exercise only.

Finally, as commonly reported in exercise studies, there appears to be a large variation in individual responses to the different exercise stimuli, which given the small number of participants in both groups this may have inflated the observed effect sizes. Therfeore, the results of the present analysis should be treated with caution and need to be confirmed in a larger number of partcipants.

6.8 Conclusions

In context of the limitations outlined above, the present study observed a *likely* beneficial increase in p-Akt following 12-weeks of regular CE in comparison to the AE group. The suppression of p-Akt following the initial bout of exercise in both groups may indicate that CKD patients exhibit an impaired response to the exercise stimulus in the untrained state. If patients exhibit a similar response to other anabolic stimuli such as feeding it may in part explain the muscle wasting experienced in this patient population. Taken together, this highlights the importance of incorporating regular resistance training in exercise/rehabilitation programmes aimed at improving muscle mass in non-dialysis CKD.

Chapter 7

The Effects of Aerobic or Combined
Exercise on Myogenic Regulatory Factor,
Inflammatory Cytokine, and Myostatin
Gene Expression in non-dialysis CKD

The recruitment, sample collection, and gene expression analysis of the non-CKD control
participants taking part in the EXPLORE CKD study presented in this chapter was
performed by Dr Emma Watson. I was responsible for performing statistical analysis and
data interpretation.

7.1 Introduction

Chronic kidney disease is a catabolic condition characterised by low grade systemic inflammation, acidosis, excess glucocorticoids, and physical inactivity, that appear to converge and stimulate muscle wasting through impairing the insulin/IGF-1 signalling pathway, which results in an imbalance between protein synthesis and degradation (discussed in previous chapters).

In addition to abnormalities in insulin/IGF-1 signalling, impaired activity of muscle satellite cells that play an important role in muscle repair and regeneration has been shown to contribute to muscle wasting in CKD (Zhang, Wang et al. 2010) (described in Chapter 2.10). The process of muscle repair and regeneration by satellite cells is controlled by the up- or down-regulation of Pax7 and the MRFs, myf5, MyoD, and myogenin (Figure 2.1) (Seale, Sabourin et al. 2000). Animal models of CKD exhibit reduced expression of Pax7 and the MRFs including MyoD and myogenin, resulting in decreased satellite cell activation, proliferation, and differentiation in response to injury, and ultimately muscle atrophy and fibrosis (Wang, Du et al. 2009, Zhang, Wang et al. 2010). However, whether human patients with non-dialysis CKD exhibit the same reductions in SC number and expression of the MRFs is currently unknown.

Importantly, exercise appears to be able to overcome abnormalities in satellite cell function in animal CKD models. In the aforementioned study, Wang and colleagues (2009) investigated work overload and treadmill running in mice with CKD. The authors reported increases in MyoD and myogenin mRNA in the muscles following work overload, but not treadmill running. In support of this, a more recent study by Molsted et al. (2015) reported increases in satellite cell number in type I fibres and myonuclear content of type II fibres of haemodialysis patients following 16-weeks of high intensity resistance exercise that resulted in muscle hypertrophy.

This finding supports numerous previous studies in healthy human subjects that report increased satellite cell number, myonuclear content, and expression of the MRFs following both acute and chronic resistance exercise (Crameri, Langberg et al. 2004, Bellamy, Joanisse et al. 2014, McKay, O'Reilly et al. 2008, Walker, Fry et al. 2012, Caldow, Thomas et al. 2015, Kadi, Thornell 2000, Roth, Martel et al. 2001). In contrast, the satellite cell response to aerobic exercise is equivocal, with some studies reporting increases in cell activation, content, and MRFs following both acute and regular aerobic exercise (Charifi, Kadi et al. 2003, Fry, Noehren et al. 2014, Joanisse, Gillen et al. 2013) whilst others report no change (Snijders, Verdijk et al. 2011). It is likely that the intensity of exercise and physical conditioning of participants will affect satellite cell activation following aerobic exercise (Nederveen, Joanisse et al. 2015, Kurosaka, Naito et al. 2012), however it is plausible that in deconditioned subjects (such as those with CKD) aerobic exercise will be a sufficient stimulus for satellite cell activation. Importantly, impairments in the insulin/IGF-1 signalling pathway (discussed previously) appear to contribute to satellite cell dysfunction in CKD (Zhang, Wang et al. 2010), whilst upregulation of IGF-1 following exercise may partly explain satellite cell activation following exercise. However, the effects of different modes of exercise on markers of satellite cell activation in non-dialysis CKD are currently unknown.

Chronic systemic elevation of IL-6 is associated with a catabolic state and has been frequently implicated in muscle wasting in a number of conditions including CKD where it results in reduced p-Akt (Zhang, Du et al. 2009), the consequences of which have been described in previous chapters. In contrast, it is well established that IL-6 is secreted from skeletal muscle following exercise and has a number of autocrine and paracrine functions (Pedersen, Febbraio 2008). Indeed, in non-dialysis CKD patients systemic IL-6 levels were immediately increased following an acute bout of walking resulting in the subsequent increase in circulating IL-10, and an anti-inflammatory environment (Viana, Kosmadakis et al. 2014).

Moreover, inflammatory cytokines, most notably IL-6 have been shown to play a significant role in SC mediated muscle hypertrophy following exercise (Bellamy, Joanisse et al. 2014, Begue, Douillard et al. 2013, Serrano, Baeza-Raja et al. 2008). In a

series of experiments Serrano et al. (2008) demonstrated that IL-6 knock-out mice exhibited a blunted hypertrophic and SC myonuclear response to overloading, in addition to reduced proliferative capacity both *in vivo* and *in vitro.* Moreover, it has been demonstrated that SCs express IL-6 and IL-6 receptor alpha which coincided with an increase in proliferating SCs following lengthening contractions in human participants (McKay, De Lisio et al. 2009). However, few studies have investigated inflammatory cytokine expression within the skeletal muscle in response to acute exercise, and how this relates to SC activation.

Myostatin is a member of the transforming growth factor-β (TGF-β) family of secreted growth factors that is predominantly expressed in skeletal muscle. Myostatin is a potent negative regulator of muscle mass, and is produced in skeletal muscle as prepromyostatin, and subsequently cleaved to produce myostatin and a propeptide (promyostatin) (Rodriguez, Vernus et al. 2014). The propeptide renders myostatin inactive by forming a latent complex. The latent complex is then activated by liberating myostatin to exert its effects regulating muscle mass and function (Rodriguez, Vernus et al. 2014). Myostatin binds to the activin IIB receptor (ActIIBR) on muscle membranes with high affinity, resulting in the activation of the Type-1 activin receptor serine kinases, ALK4 or ALK5. These kinases subsequently phosphorylate Smad 2 and 3 proteins factors that function as key intracellular mediators for myostatin signalling as they translocate to the nucleus and initiate transcription of its target genes (Han, Zhou et al. 2013).

Myostatin was first identified as a potent regulator of muscle mass by McPherron and colleagues (1997) where genetic depletion of myostatin resulted in gross muscle hypertrophy. Moreover, since its discovery elevated levels of myostatin have been observed in muscle wasting and have been reported in a number of catabolic conditions including CKD (Avin, Chen et al. 2016, Dong, Dong et al. 2017, Zhang, Rajan et al. 2011). Evidence from mouse models of CKD have shown that myostatin reduces signalling through the insulin/IGF1 pathway and inhibits satellite cell function and proliferation which contributes to muscle atrophy and fibrosis (Zhang, Rajan et al. 2011, Wang, Mitch 2014, Dong, Dong et al. 2017). Interestingly, these effects appear to be prevented with the inhibition of myostatin (Zhang, Rajan et al. 2011). Exercise may be

beneficial in reducing myostatin expression in skeletal muscle in CKD populations. A study by Sun et al. (2006) conducted in an animal model of CKD reported reductions in myostatin gene expression following work overload. Similarly, reductions in myostatin gene expression have been reported in haemodialysis patients following regular exercise consisting of aerobic and resistance exercise performed alone or in a combined programme (Kopple, Wang et al. 2007). However, the effects of exercise on myostatin signalling in the skeletal muscle of non-dialysis CKD patients are currently unknown.

7.2 Aims and hypotheses

The aim of this chapter was to examine the expression of MRFs, myostatin, and inflammatory cytokines in the skeletal muscle of non-dialysis CKD and investigate the response to acute and prolonged aerobic or combined exercise by addressing the following hypotheses:

7.2.1 Expression of myogenic regulatory factors

- It was hypothesised that the mRNA expression of the MRFs would be lower in the skeletal muscle of CKD participants compared with healthy controls.

- Both modes of exercise would increase the expression of MRFs following unaccustomed and accustomed exercise training, however the increase would be greater in those performing CE.

7.2.2 Myostatin and ActIIBR expression

- Myostatin and ActIIBR gene expression would be increased in the skeletal muscle of CKD participants compared with healthy controls
- Myostatin and the ActIIBR would be reduced following unaccustomed and regular exercise in both AE and CE groups.

7.2.3 Inflammatory cytokines

- The gene expression of the inflammatory cytokines would be greater in the skeletal muscle of CKD participants versus healthy control participants.

- Both AE and CE would increase the expression of IL-6 and associate with the expression of the MRFs.

7.3 Methods

7.3.1 CKD participants

7.3.1.1 Recruitment

Participants included in this chapter are a sub group of those who successfully completed the ExTra CKD trial (ISRCTN registry registration number: 36489137) described fully in Chapter 3.

7.3.1.2 Exercise interventions

Both exercise interventions are described fully in Chapter 3.

7.3.1.3 Muscle biopsy sampling and handling

A full description of the muscle biopsy and sample handling procedures is provided in Chapter 3. Briefly, biopsies were obtained at baseline, 24h after the first (to investigate the acute effects of exercise in an 'untrained' state), and final (to investigate the effect of regular exercise in the 'trained' state) exercise sessions from the middle portion of the vastus lateralis muscle of the individuals' right leg using a 12-gauge microbiopsy needle. Biopsies samples were immediately snap-frozen and stored in liquid nitrogen until analysis.

7.3.1.4 Muscle biopsy analysis

A detailed description of all methods used in biopsy analysis is provided in Chapter 3. Biopsies were obtained at baseline (B1), and then 24h after the first (B2), and final (B3) exercise sessions, to investigate the acute response to exercise in the untrained and trained states, respectively.

7.3.2 Non-CKD control participants

7.3.2.1 Recruitment

Resting muscle samples of age matched healthy individuals who were taking part in the EXPLORE CKD study (ISRCTN registry registration number: 18221837) were included as a comparison against CKD samples for the expression of the genes of interest at baseline. A one off resting muscle sample was collected from healthy individuals who were scheduled to undergo routine orthopaedic surgery at the Leicester General Hospital, or from individuals known to the study team i.e. CKD patient spouses. Participants recruited from surgery were approached by their consultant surgeon at their pre-operation assessment visit and consented by the researcher on the day of their operation. All other participants were approached by a member of the study team and given at least 48 hours to consider the PIS before informed consent was taken on the day of the biopsy. The study received approval from the National Research Ethics Committee, East Midlands-Northampton (Ref 13/EM/0344) and all patients gave informed consent.

7.3.2.2 Eligibility and exclusion criteria

Healthy control participants were initially verbally screened for eligibility to assess if they met any of the following exclusion criteria; age <18 years, diagnosed CKD (eGFR <80 mL/min/1.73m^2), presence of catabolic illness (e.g. cancer, sepsis, burns, and HIV), uncontrolled diabetes mellitus (HbA1c >9%), or an inability to give informed consent. To confirm participant eligibility, a blood sample was collected one week before the surgery during pre-assessment clinic to measure eGFR.

7.3.2.3 Muscle biopsy procedures

For participants who were already known to the study team the biopsy procedure was the same as previously outlined for CKD participants (see Chapter 3). For healthy control participants undergoing routine orthopaedic surgery within the region of the quadriceps, an open biopsy procedure was conducted, where by the surgeon removed a small amount of muscle tissue using from the existing open site using a surgical blade. The tissue was then dissected into smaller pieces with a small piece of tissue being placed immediatley into *RNAlater*® (Sigma, UK), and stored overnight at 4°C before being transferred to -80°C for longterm storage. RNA was extracted as outlined previously (section 3.14.1).

7.3.3 Determination of MRFs, myostatin, and cytokine mRNA expression by quantitative RT-PCR

The mRNA expression of Pax7 and three of the MRFs (Myf5, MyoD, and myogenin), myostatin and its ActRIIB, and inflammatory cyotkines (IL-6, TNFα, and MCP-1) were determined using qRT-PCR following the procedure described previously (Chapter 3). The Ct values from the genes of interest were normalized to the Ct values of 18S as the reference gene. Fold changes in gene expression following exercise were calculated using the $2^{-\Delta\Delta Ct}$ method which sets the baseline value to 1.0. For the comparison between CKD and healthy controls relative gene expression was calculated as $2^{-\Delta Ct}$. Primers and probes for Pax7 (Hs00242962_m1), Myf5 (Hs00929416_g1), MyoD (Hs02330075_g1), myogenin (Hs01072232_m1), IL-6 (Hs00985639_m1), TNFα (Hs01113624_g1), MCP-1 (Hs00234140_m1), myostatin (Hs00976237_m1), activin receptor IIB (Hs00155658_m1), and 18S (Hs99999901_s1) were supplied as TaqMan gene expression assays (Applied Biosystems, Warrington, UK).

7.3.4 Determination of Smad 2/3 phosphorylation in response to exercise

Phosphorylation status of Smad2 (ser465/467)/ Smad3 (ser423/425) and total Smad2/3 protein content in response to AE and CE was measured using western blotting as previously described in Chapter 3. The content of phosphorylated Smad2/3 and total Smad2/3 are presented relative to GAPDH as a loading control.

7.3.5 Statistical analysis

The distribution of the data was assessed using the Shapiro-Wilkes test. Variables that were not normally distributed were log-transformed for analysis, and back transformed for presentation as mean ± SD. Where log-transformation did not restore the normal distribution, non-parametric analysis was performed and data presented as the median [IQR]. Mann-Whitney U tests were performed to compare baseline gene expression between CKD and control participants.

To investigate the effects of exercise on gene expression in CKD, analysis was performed as described in previous chapters. Linear mixed models were carried out using IBM SPSS Statistics (IBM, Chicago, IL) Version 24, whilst effect sizes and MBI were calculated using custom Microsoft Excel spreadsheets (Hopkins 2007). Statistical significance was accepted at $P < 0.05$.

7.4 Results

7.4.1 Participant demographics

The same 19 participants with CKD (described in Chapter 6) who completed the ExTra CKD study and consented to give multiple muscle biopsies are included in the analysis for the present chapter (demographics are displayed in Table 7.1). In addition 11 healthy participants consented to give a one off muscle biopsy to allow for a comparison with 11 age- and sex-matched CKD participants. The demographics of the non-CKD control and CKD participants included in the comparison are displayed in Table 7.1.

Table 7.1 Demographics of participants included in the non-CKD control and CKD comparisons

	Control	CKD
Number of participants	11	11
Age (years)	63 ± 10	63 ± 11
Sex (*n* men/women)	5/6	5/6
eGFR (mL/min/1.73m^2)	76 ± 8	26 ± 7

Data presented as mean ± SD. Abbreviations: eGFR = estimated glomerular filtration rate.

7.4.2 Comparison between CKD and control participants

Results of the comparison between control and CKD participants are displayed in Table 7.2. The expression of all genes tended to be higher in CKD participants; notably, myostatin and the ActIIBR were substantially upregulated by 71.6 and 18.8 fold, respectively (Figure 1 A and B). Moreover, the mRNA expression of TNF-α was also substantially upregulated by 8.7 fold, whilst IL-6 expression tended to be elevated by a median of 5.9-fold (Figure 7.1 C and D). An independent samples t-test revealed no systematic differences ($p=.238$) in the mean gene expression of 18S between control (mean Ct 11.92 ± .14) and CKD (mean Ct 12.55 ± 1.65) tissue.

Table 7.2 Median fold change in CKD participants vs. controls

Gene	CKD median fold change	*P*
Myogenic regulators		
Pax7	↑ 1.8	.657
Myf5	↑ 2.8	.847
MyoD	↑ 5.4	.605
Myogenin	↑ 3.0	.659
Myostatin signalling		
Myostatin	↑ 71.6	**.016***
ActIIBR	↑ 18.8	**.020***
Inflammatory cytokines		
IL-6	↑ 5.9	.056
TNF-α	↑ 8.7	**<.001***
MCP-1	↑ 1.3	.400

Abbreviations: Pax7 = paired box transcription factor; Myf5 = myogenic factor 5; ActIIBR = activin IIB receptor; IL-6 = interleukin-6; TNF-α = tumour necrosis factor-alpha; MCP-1 = monocyte chemoattractant-1.

Figure 7.1 – Comparison between control and CKD participants. Graphs show median (95% CI) for relative expression, presented as $2^{-\Delta Ct} \times 10^5$. (A) Myostatin mRNA expression (71.6-fold higher in CKD). (B) Activin IIB receptor mRNA expression (18.8-fold higher in CKD). (C) TNF-α mRNA expression (8.1-fold higher in CKD). (D) IL-6 mRNA expression (5.9-fold higher in CKD).

* Denotes $p < 0.05$ from control values.

7.4.3 Effects of aerobic or combined exercise in CKD

7.4.3.1 Myogenic regulatory factors

In response to exercise, Pax7 exhibited a large effect size for time ($F=3.296$, $\eta p^2=.171$, $p=.050$), but not group x time interactions ($F=.308$, $\eta p^2=.019$, $p=.737$). Between group comparisons for the effects of exercise on Pax7 gene expression were deemed *unclear* using MBI analysis, with AE and CE exhibiting similar mean changes following unaccustomed and accustomed exercise (Table 7.3). Similarly, no time or group x time

interactions were obsered for Myf5 mRNA expression, with modest fold changes from baseline in both groups that were deemed *unclear*.

In contrast, MyoD exhibited large effect sizes and significant time (F=5.795, ηp^2=.254, p=.016) and group x time interactions (F=4.630, ηp^2=.214, p=.031). MBI analysis (Table 7.3) revealed that the change in MyoD gene expression following unaccustomed CE was *likely* (84% liklihood of at least a small effect) reduced below that of the AE group (Table 7.3). Indeed, unaccustomed AE appeared to exert minimal effect on MyoD gene expression (mean fold-change 1.00 CI 0.29 to 3.62) whilst unaccustomed CE resulted in a mean reduction from baseline (mean fold-change 0.29 (-71%) CI 0.11 to 0.79). Following accustomed CE a small mean increase (1.54-fold CI 0.97 to 2.5) was observed, however the between group effects were deemed *unclear*. The change in MyoD gene expression between the unaccustomed and accustomed response was deemed *likely* (92% likelihood of at least small effect) increased in the CE group compared to AE. Figure 7.2 depicts the MyoD mRNA responses following AE and CE.

No significant interactions for time (F=.549, ηp^2=0.28, p=.615) or group x time (F=2.323, ηp^2=.120, p=.113) were observed for the effects of exercise on myogenin gene expression. However, when adjusting for baseline values MBI analysis revealed the change in myogenin gene expression following unaccustomed CE was *likely* (84% likelihood of at least small effect) reduced compared to AE. Whilst the change in myogenin expression was deemed unclear between groups following accustomed exercise, the difference between unaccustomed and accustomed was deemed *likely* (85% likelihood of at least small effect) increased in those performing CE (Table 7.3).

Figure 7.2 - Changes in MyoD mRNA expression following AE and CE in CKD participants. Graphs A and B show mean relative expression MyoD mRNA with 90% CI following aerobic and combined exercise, respectively. Individual changes in relative expression of MyoD following aerobic (C) and combined exercise (D). Baseline is represented by the broken horizontal line and a value of 1.

# Denotes $p < .05$ from untrained response

Figure 7.3 Changes in myogenin gene expression following AE and CE in CKD participants. Graphs A and B show mean relative expression myogenin mRNA with 90% CI following aerobic and combined exercise, respectively. Individual changes in relative expression of myogenin following aerobic (C) and combined exercise (D). Baseline is represented by the broken horizontal line and a value of 1.

Table 7.3 Expression of the myogenic regulatory factors following aerobic or combined exercise in CKD

Gene	Time point	Aerobic Fold-Change (90% CI)	p	Combined Fold-Change (90% CI)	p	AE vs. CE Fold-Change in CE (90% CI)	d (90% CI)	Inference
Pax7	B1-B2	0.56 (0.28 to 1.1)	.169	0.46 (0.2 to 1)	.113	0.95 (0.82 to 1.07)	-.22 (-.34 to .77)	*Unclear*
	B1-B3	0.81 (0.49 to 1.4)	.469	1.05 (0.44 to 2.5)	.920	1.00 (0.88 to 1.11)	.17 (-.56 to .57)	*Unclear*
	B2-B3	1.46 (0.61 to 3.5)	.445	2.30 (1.3 to 4)	**.025**	1.04 (0.97 to 1.12)	-.27 (-.73 to .20)	*Possibly ↑ (44%)*
Myf5	B1-B2	1.27 (0.37 to 4.4)	.733	0.78 (0.45 to 1.4)	.429	0.95 (0.84 to 1.06)	-.24 (-.81 to .32)	*Unclear*
	B1-B3	0.84 (0.46 to 1.5)	.606	1.14 (0.57 to 2.3)	.732	1.02 (0.97 to 1.07)	.17 (-.25 to .59)	*Unclear*
	B2-B3	0.66 (0.22 to 1.9)	.499	1.46 (0.63 to 3.4)	.426	1.06 (0.97 to 1.15)	-.39 (-.96 to .19)	*Unclear*
MyoD	B1-B2	1.00 (0.29 to 3.62)	.988	0.29 (0.11 to 0.79)	.050	0.89 (0.79 to 1.01)	-.46 (-.05 to -.88)	*Likely ↓ (84%)*
	B1-B3	1.14 (0.8 to 1.6)	.511	1.54 (0.97 to 2.5)	.123	1.02 (0.98 to 1.07)	.14 (-.12 to .39)	*Unclear*
	B2-B3	1.13 (0.61 to 2.1)	.728	5.29 (2 to 14)	**.012**	1.12 (1.04 to 1.19)	.60 (.18 to 1.01)	*Likely ↑ (92%)*
Myogenin	B1-B2	1.27 (0.57 to 2.8)	.599	0.42 (0.14 to 1.3)	.193	0.49 (0.65 to 1.01)	-.42 (-.89 to .05)	*Likely ↓ (84%)*
	B1-B3	0.80 (0.54 to 1.2)	.326	0.90 (0.42 to 1.9)	.803	1.02 (0.89 to 1.17)	.08 (-.39 to .55)	*Unclear*
	B2-B3	0.63 (0.31 to 1.3)	.267	2.14 (0.79 to 5.8)	.195	1.11 (1.01 to 1.22)	.43 (.03 to .82)	*Likely ↑ (85%)*

Key: Time point = biopsy time point where; B1-B2 = change in concentration from baseline in response to acute exercise in the untrained state, B1-B3 = change in concentration from baseline in response to acute exercise in the trained state, B2-B3 = difference between response in untrained and trained states; Inference = qualitative inference (likelihood of at least small effect %). Abbreviations: Pax7 = paired box transcription factor; Myf5 = myogenic factor 5; MyoD = myogenic differentiation 1. d = effect size where; 0.2 = small, 0.5 = moderate, and >0.8 = large effect

7.4.3.2   Myostatin gene expression and downstream signalling

A large effect and interaction for time (F=11.098, ηp^2=.581, p=.001) but not group x time (F=.163, ηp^2=.020, p=.851) was observed for myostatin gene expression. Both AE and CE demonstrated mean reductions in myostatin gene expression following unaccustomed and accustomed exercise, with between group differences that were deemed *unclear* when adjusting for baseline values. Changes in myostatin gene expression are shown in Figure 7.3.

ActIIBR mRNA expression exhibited a large effect for time (F=2.971, ηp^2=.149, p=.065) but not group x time (F=.511, ηp^2=.029, p=.604). Similar to myostatin, both groups exhibited mean reductions from baseline levels following both unaccustomed and accustomed AE and CE, however the difference in effects between the two groups were deemed *unclear*. Whilst the response of the ActIIBR appeared to be similar to that of myostain, no associations between the expression changes of the two genes were observed.

Despite mean reductions in myostatin gene expression following both AE and CE, no interactions and small effect sizes for time (F=.772, ηp^2=.049, p=.471) or group x time (F=.391, ηp^2=.025, p=.680) for its downstream target, Smad2/3 phosphorylation were seen. Indeed all changes in p-Smad2/3 between AE and CE were deemed *unclear* (Table 7.4).

Table 7.4 Gene expression of myostatin and activin IIB receptor, and Smad 2/3 phosphorylation following aerobic or combined exercise in CKD

Gene/ Protein	Time point	Aerobic		Combined		AE vs. CE		
		Fold-Change (90%CI)	*p*	Fold-Change (90%CI)	*p*	Fold-Change in CE (90% CI)	*d* (90% CI)	Inference
Myostatin	B1-B2	0.16 (0.05 to 0.50)	**.016**	0.16 (0.06 to 0.40)	**.006**	1.12 (0.90 to 1.38)	.25 (-.22 to .77)	*Unclear*
	B1-B3	0.38 (0.18 to 0.81)	**.044**	0.57 (0.21 to 1.5)	.324	1.00 (0.86 to 1.16)	-.01 (-.31 to .30)	*Unclear*
	B2-B3	2.24 (0.55 to 9.1)	.320	3.65 (1.79 to 7.9)	**.014**	1.11 (0.92 to 1.32)	.27 (-.19 to .74)	*Possibly ↑ (61%)*
ActIIBR	B1-B2	0.69 (0.32 to 1.5)	.403	0.39 (0.18 to 0.84)	.051	0.92 (0.77 to 1.08)	.32 (-.18 to .81)	*Possibly ↓ (67%)*
	B1-B3	0.70 (0.41 to 1.2)	.253	0.48 (0.19 to 1.2)	.177	0.95 (0.09 to 1.09)	.22 (-.29 to .72)	*Unclear*
	B2-B3	1.00 (0.59 to 1.7)	.992	1.23 (0.61 to 2.5)	.599	1.03 (0.94 to 1.11)	.13 (-.54 to .28)	*Unclear*
pSmad2/3	B1-B2	26% (-15 to 90)	.302	0% (-14 to 156)	.610	-29 (-75 to 104)	-.33 (-1.4 to .69)	*Unclear*
	B1-B3	57% (-61 to 630)	.567	-41% (-88 to 280)	.550	-42 (-82 to 86)	-.46 (-1.5 to .54)	*Unclear*
	B2-B3	25% (-48 to 300)	.650	-41% (-77 to 150)	.321	-18 (-69 to 119)	-.19 (-1.1 to .77)	*Unclear*

Key: Time point = biopsy time point where; B1-B2 = change in concentration from baseline in response to acute exercise in the untrained state, B1-B3 = change in concentration from baseline in response to acute exercise in the trained state, B2-B3 = difference between response in untrained and trained states; Inference = qualitative inference (likelihood of at least small effect %). Abbreviations: ActIIBR = activin IIB receptor; pSmad 2/3 = phosphorylated Smad2/3. *d* = effect size where; 0.2 = small, 0.5 = moderate, and >0.8 = large effect.

Figure 7.4 - Changes in Myostain mRNA expression following AE and CE in CKD participants. Graphs A and B show mean relative expression myostatin mRNA with 90% CI following aerobic and combined exercise, respectively. Individual relative expression of myostatin following aerobic (C) and combined exercise (D). The broken horizontal line and a value of 1 represent baseline.

* Denotes *p*<.05 from baseline

# Denotes *p*<.05 from untrained response

Aerobic   **Combined**

A   B

C   D

Figure 7.5 - Changes in activin IIB receptor mRNA expression following AE and CE in CKD participants. Graphs A and B show mean relative expression ActIIBR mRNA with 90% CI following aerobic and combined exercise, respectively. Individual relative expression of ActIIBR following aerobic (C) and combined exercise (D). The broken horizontal line and a value of 1 represent baseline.

7.4.3.3   Inflammatory cytokines

The mRNA expression of the inflammatory cytokines IL-6, TNFα, and MCP-1 all exhibited similar responses to both AE and CE in the untrained and trained states, where significant interactions and large effect sizes for time were observed, but not group x time. *Post hoc* analysis displayed in Table 7.5, showed substantial increases in the gene expression of all cytokines in response to unaccustomed exercise, with the between group effects deemed *unclear* using MBI analysis. Similarly, following 12-weeks of regular exercise changes in gene expression between groups were deemed *unclear* or *trivial*.

Figure 7.6 - Changes in IL-6 mRNA expression following AE and CE in CKD participants. Graphs A and B show mean relative expression of IL-6 mRNA with 90% CI following aerobic and combined exercise, respectively. Individual relative expression of IL-6 following aerobic (C) and combined exercise (D). The broken horizontal line and a value of 1 represent baseline.

* Denotes *p*<.05 from baseline

# Denotes *p*<.05 from untrained response

Figure 7.7 - Changes in TNF-α receptor mRNA expression following AE and CE in CKD participants. Graphs A and B show mean relative expression TNF-α mRNA with 90% CI following aerobic and combined exercise, respectively. Individual relative expression of TNF-α following aerobic (C) and combined exercise (D). The broken horizontal line and a value of 1 represent baseline.

* Denotes *p*<.05 from baseline

# Denotes *p*<.05 from untrained response

Aerobic

Combined

Figure 7.8 - Changes in MCP-1 mRNA expression following AE and CE in CKD participants. Graphs A and B show mean relative expression MCP-1 mRNA with 90% CI following aerobic and combined exercise, respectively. Individual relative expression of MCP-1 following aerobic (C) and combined exercise (D). The broken horizontal line and a value of 1 represent baseline.

* Denotes $p<.05$ from baseline

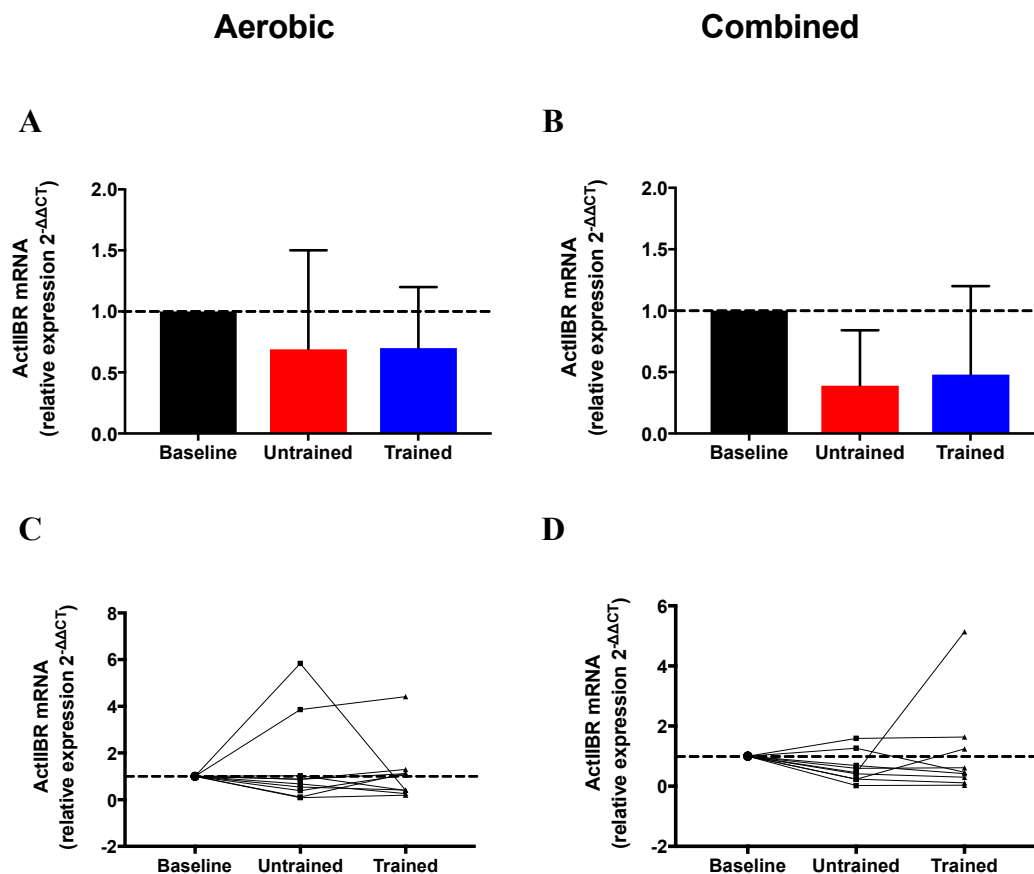\# Denotes $p<.05$ from untrained response

Table 7.5 mRNA expression of pro-inflammatory cytokines following aerobic and combined exercise in CKD

Gene	Time point	Aerobic Fold-Change (90%CI)	*p*	Combined Fold-Change (90%CI)	*p*	AE vs. CE Fold-Change in CE (90% CI)	*d* (90% CI)	Inference
IL-6	B1-B2	8.90 (2 to 40)	**.027**	5.30 (2.9 to 9.8)	**.001**	0.93 (0.76 to 1.08)	-.36 (-1.1 to .42)	*Unclear*
	B1-B3	0.72 (0.49 to 1.1)	.160	0.72 (0.41 to 1.3)	.306	1.00 (0.96 to 1.05)	.03 (-.30 to .37)	*Unclear*
	B2-B3	0.08 (0.02 to 0.4)	**.023**	0.14 (0.07 to 0.29)	**.001**	1.07 (0.90 to 1.80)	.34 (-.52 to 1.2)	*Unclear*
TNF-α	B1-B2	2.93 (1.7 to 5.1)	**.007**	1.93 (1 to 3.6)	.086	0.97 (0.91 to 1.03)	-.26 (-.74 to .23)	*Unclear*
	B1-B3	1.87 (0.58 to 6)	.350	0.89 (0.49 to 1.6)	.726	0.94 (0.83 to 1.04)	-.46 (-1.2 to .32)	*Unclear*
	B2-B3	0.64 90.23 to 1.8)	.449	0.46 (0.26 to 0.8)	**.032**	0.97 (0.87 to 1.07)	-.21 (-.85 to .44)	*Unclear*
MCP-1	B1-B2	4.61 (1.5 to 14)	**.033**	6.99 (2 to 24)	**.019**	1.03 (0.85 to 1.25)	0.09 (-.45 to .62)	*Unclear*
	B1-B3	0.73 (0.38 to 1.4)	.404	0.88 (0.52 to 1.5)	.654	1.02 (0.96 to 1.08)	0.08 (-.34 to .18)	*Trivial* (79%)
	B2-B3	0.16 (0.05 to 0.53)	**.022**	0.13 (0.05 to 0.34)	**.004**	0.95 (0.77 to 1.78)	-.14 (-.75 to .47)	*Unclear*

Key: Time point = biopsy time point where; B1-B2 = change in concentration from baseline in response to acute exercise in the untrained state, B1-B3 = change in concentration from baseline in response to acute exercise in the trained state, B2-B3 = difference between response in untrained and trained states; Inference = qualitative inference (likelihood of at least small effect %). Abbreviations: IL-6 = Interleukin-6; TNF-α = tumour necrosis factor-alpha; MCP-1 = monocyte chemoattractant-1. *d* = effect size where; 0.2 = small, 0.5 = moderate, and >0.8 = large effect.

7.5 Discussion

7.5.1 Myogenic regulatory factors

Animal models of CKD exhibit satellite cell dysfunction and reduced expression of the MRFs Myf5, MyoD and myogenin (Zhang, Wang et al. 2010, Wang, Du et al. 2009). In contrast, the data presented above shows no evidence of a reduction in the gene expression of the MRFs in the skeletal muscle of non-dialysis CKD patients. Indeed, the mRNA expression of all MRFs measured tended to be higher than in age- and sex-matched non-CKD controls (Table 7.2). Aside from species differences, a possible reason for the discrepancy between the present data and previous animal studies could be due to the severity of kidney disease in these models. Indeed, the studies conducted by Professor Mitch's group (Wang, Du et al. 2009, Zhang, Wang et al. 2010) were carried out in animals that had undergone subtotal nephrectomy that reduces kidney function by approximately 90% (Zhang, Kompa 2014), and therefore more closely replicates ESRD. In support of this notion, Avin et al (2016) used a progressive model of CKD in rats and reported reduced Pax7 expression with concomitant increases in MyoD and myogenin in the skeletal muscle in comparison with control animals.

Exercise, particularly resistance exercise is a potent stimulus for satellite cell activation and has been shown to increase satellite cell content in the skeletal muscle of haemodialysis patients (Molsted, Andersen et al. 2015). The increase in satellite cell cell activation following exercise is accompanied by increased expression of Pax7 and the MRFs which are upregulated shortly after exercise and remain elevated for >24h (Raue, Slivka et al. 2006, Caldow, Thomas et al. 2015, Yang, Creer et al. 2005). Contrary to what was hypothesised, no substantial increases were observed in the expression of the MRFs following unaccustomed or accustomed exercise in either group. Interestingly, MBI analysis revealed that the change in MyoD expression following unaccustomed CE was *likely decreased* below that of the AE group. This appears to be due to a reduction in MyoD expression following CE with a mean fold-change of 0.29 (CI 0.11 to 0.79) rather than an increase following AE exercise. This may have been partially restored following of 12-weeks of regular CE where MyoD gene expression exhibited a mean fold-change

of 1.54 (CI 0.97 to 2.5) from baseline levels. However, a large proportion of the mean increase from baseline appears to be attributed to one participant (Figure 7.2).

Following activation and proliferation satellite cells begin to express myogenin, which mediates myoblast differentiation into structural proteins (Snijders, Nederveen et al. 2015), which is also increased in the 24h following resistance exercise in healthy individuals (Kim, Kosek et al. 2005, Bickel, Slade et al. 2005). Again, myogenin expression was deemed *likely decreased* following unaccustomed CE in comparison to AE. However, as with MyoD expression, this may have been partially restored following 12-weeks of regular CE, however the between group effects following accustomed exercise were deemed unclear. These results are in line with previous work from our group where the expression of MyoD and myogenin were not increased 24h following acute unaccustomed resistance, or following 8-weeks of regular resistance exercise (Watson, Viana et al. 2017). Whilst no differences between the expression of the MRFs between CKD and healthy individuals were observed in the present chapter, the fact that there was an inability to upregulate these genes following acute exercise may suggest possible impairments in the factors responsible for satellite cell activation, proliferation and differentiation in response to exercise in CKD.

It is plausible that the possible impairments in MRF function following exercise observed here are associated with the abnormal IGF-1/Akt signalling following exercise reported in the previous chapter. Such impairments in satellite cell activity have previously been reported in animal models of CKD (Zhang, Wang et al. 2010) where the authors concluded that CKD results in suppressed p-Akt in satellite cells following injury, resulting in reduced expression of the MRFs and abnormalities in cell activation and proliferation. Indeed, satellite cells co-express IGF-1 following damaging exercise (McKay, O'Reilly et al. 2008), which plays an important role in their progression through controlling the expression of the MRFs (Snijders, Nederveen et al. 2015). In contrast to the results presented here and in the previous chapter, MRF expression and impaired IGF-1/Akt signalling (as previously discussed) appears to be restored following chronic overload (Wang, Du et al. 2009) in animal models of CKD. Again methodological differences may explain the discrepant findings between the studies in humans and

animals with CKD. In the overload model used by Wang and colleagues (2009) the plantaris muscle was placed under constant overload for a 2-week period by the removal of gastrocnemius, in comparison to resistance exercise in humans, which is characterised by transient bouts of short high velocity contractions. Moreover, as described in the previous chapter, the present analysis is limited by the biopsy timing and only provides a snapshot, which may have meant changes in the expression of the MRFs have been missed. Further research is required to confirm if CKD patients exhibit an impaired SC response to exercise, and if so, the mechanisms underpinning this.

7.5.2   Myostatin signalling

Myostatin is well recognised as a potent negative regulator of muscle mass and has frequently been implicated in the muscle wasting associated with CKD. In skeletal muscle, myostatin binds to the ActIIBR and exerts its downstream effects that include inhibition of protein synthesis, and concurrent increases in catabolism, and suppression of MRF expression (Han, Zhou et al. 2013). In agreement with this, both myostatin and its receptor, ActIIBR were substantially upregulated in the skeletal muscle of CKD patients compared to age- and sex-matched non-CKD controls, however this does not appear to be associated with reduced expression of the MRFs as discussed above. Elevated mRNA expression of myostatin has previously been reported in animal models of CKD (Avin, Chen et al. 2016, Sun, Chen et al. 2006), and patients at the onset of peritoneal dialysis (Verzola, Procopio et al. 2011). However, to the best of my knowledge this is the first report of elevated myostatin and ActIIBR mRNA in CKD patients with earlier stages of CKD.

An important finding in the present study was the suppression of myostatin expression following acute exercise. As hypothesised, both AE and CE substantially reduced myostatin expression below baseline levels by 84% following unaccustomed exercise. This response remained following 12-weeks AE, however in those performing CE despite 6 out of the 9 participants in this group showing reductions in myostatin expression, the mean expression was upregulated from the unaccustomed response. No between group differences were observed following unaccustomed or accustomed exercise where the

effects were deemed *unclear.* Similar favourable effects of exercise have previously been reported following work overload in an animal model of ESRD (Sun, Chen et al. 2006) and a combination of exercise programmes involving aerobic and resistance exercise in haemodialysis patients (Kopple, Wang et al. 2007). Moreover, recent work by our group reported reductions in myostatin mRNA in the 24h following both unaccustomed and 8-weeks of regular resistance exercise (Watson, Viana et al. 2017). When taking the results of the present and previous data in both dialysis dependent and independent CKD, it appears that exercise of varying modes is effective at suppressing myostatin expression.

Whilst myostatin negatively regulates satellite cell activation (McCroskery, Thomas et al. 2003) the reduction in myostatin gene expression here was not associated with an upregulation of MyoD indicating no change in satellite cell activation. Moreover, whilst myostatin inhibition induces myofibre hypertrophy in animals (Zhang, Rajan et al. 2011, Wang, McPherron 2012), the downregulation of myostatin mRNA following exercise does not appear to be required to promote hypertrophy in healthy humans (Kim, Petrella et al. 2007). Despite this, a reduction in myostatin expression is likely to exert other beneficial effects given its role in other muscle pathologies (Dong, Dong et al. 2017, Zhang, Rajan et al. 2011). Further research is required to determine the importance of myostatin suppression following exercise in CKD.

7.5.3 Inflammatory cytokines

Pro-inflammatory cytokines have been implicated in the muscle wasting process in CKD (Wang, Mitch 2014). In line with this, the present data provides evidence for increased expression of pro-inflammatory cytokines in the skeletal muscle of non-dialysis CKD patients. In comparison to non-CKD controls the mRNA expression of TNF-α was substantially upregulated by a median of 8.7-fold, whilst IL-6 was elevated by a median of 5.9-fold. The up-regulation of pro-inflammatory genes has previously been observed in the skeletal muscle of animals and patients with ESRD where they contribute to local systemic inflammation (Garibotto, Sofia et al. 2006), however, this is the first report of increased mRNA expression of inflammatory cytokines in the earlier stages of CKD. Importantly, in animal models of CKD both TNF-α and IL-6 are capable of stimulating

muscle protein catabolism through suppression of p-Akt (Thomas, Dong et al. 2013, Zhang, Du et al. 2009, Zhang, Rajan et al. 2011). Moreover, Wang and colleagues (2015) demonstrated that elevated levels of TNF-α resulted in the up-regulation of myostatin expression and subsequent atrophy in C2C12 myotubes in a nuclear-factor kappaB (NF-kB) dependent pathway. In support of this, a recent study by Verzola and colleagues (2017) identified elevated toll-like receptor 4 expression in skeletal muscle of patients with CKD stage 5 which is capable of directing the production of TNF-α through NF-kB signalling. However, no associations were observed between the mRNA expression of TNF-α or IL-6 with myostatin in the present analysis.

No between group differences were observed between the effects of AE or CE on the inflammatory cytokines measured following unaccustomed or accustomed exercise. As hypothesised, both unaccustomed AE and CE resulted in substantial mean increases in the mRNA expression of IL-6, TNF-α, and MCP-1. This is consistent with previous studies in healthy individuals that report marked increases in the gene expression of TNF-α, MCP-1, and IL-6 following both aerobic and resistance exercise performed alone or in combination (Louis, Raue et al. 2007, Mathers, Farnfield et al. 2012, Donges, Duffield et al. 2014). After 12-weeks of regular training in both groups the increase in pro-inflammatory cytokine expression was attenuated, which suggests an adaptive mechanism to regular exercise. Again, this finding is in line with previous work from our group following regular resistance exercise, where pro-inflammatory cytokines were upregulated 24h following unaccustomed resistance exercise, but the effect was diminished following 8-weeks of training (Watson, Viana et al. 2017).

Whilst skeletal muscle produces and secretes cytokines, most notably IL-6, in response to exercise (Pedersen, Febbraio 2008), the mRNA expression and release of IL-6 from the muscle peaks shortly following cessation of the bout of exercise and returns to pre-exercise levels shortly afterwards (Fischer, Hiscock et al. 2004, Pedersen, Febbraio 2008). Therefore the increase in cytokine expression following unaccustomed exercise may be a result of muscle damage as described in the previous chapter, with invadinding immune cells being the source of the mRNA expression of the inflammatory cytokines. Indeed, muscle damage such as that which occurs following unaccustomed exercise

initiates an inflammatory response characterised by the infiltration of neutrophils following the release of pro-inflammatory cytokines and chemoattractants from resident macrophages (Tidball 2017). Notably the release of chemoattractants includes MCP-1, which plays an important role mediating muscle inflammation and regeneration following injury through attracting immune cells to the site of injury (Shireman, Contreras-Shannon et al. 2007, Lu, Huang et al. 2011). The rapid invasion of neutrophils promotes the removal of damaged proteins and cell debris, and the secretion of pro-inflammatory cytokines, which stimulates the infiltration of circulating monocytes and macrophages to the site of injury (Martinez, McHale et al. 2010). This further promotes a pro-inflammatory environment through the release of pro-inflammatory cytokines including TNF-α (Novak, Weinheimer-Haus et al. 2014), which appear to play a role in modulating the MRFs including MyoD (Warren, Hulderman et al. 2002).

Moreover, IL-6 also appears to be important for satellite cell mediated muscle hypertrophy, through STAT3 signalling. In line with this and the data presented in this chapter, increased expression of IL-6 is observed in the skeletal muscle and satellite cells of healthy individuals 24h following acute damaging exercise in healthy individuals (Toth, McKay et al. 2011, McKay, De Lisio et al. 2009). However, in contrast to the present study, the increase in IL-6 in the above mentioned studies occurred with a concomitant increase in satellite cells expressing Pax7 at 24h post exercise, which continued to increase until 72h following exercise. As discussed above, in the present study the gene expression of the MRFs showed no change from baseline levels in response to unaccustomed exercise, which may indicate an inability for satellite cells to be activated and proliferate. Unfortunately, the single biopsy at 24h means it is not possible to rule out changes in the time course of satellite cell activation in response to exercise.

Whilst it is hypothesised that the increases in pro-inflammatory cytokines following both unaccustomed AE and CE represent an immune response to muscle damage, the effects of increasing already elevated IL-6 and TNF-α gene expression is unknown. However, a heightened and prolonged immune response to muscle damage has previously been reported in an animal model of CKD (Zhang, Wang et al. 2010). The authors reported

increased prolonged mononuclear cell accumulation in addition to increased expression of inflammatory cytokines and chemokines in injured muscles in comparison to control animals. It is possible that the increased expression of already elevated pro-inflammatory cytokines observed in the present study may indicate a similar heightened inflammatory response to muscle damage in human CKD patients, which may result in delayed skeletal muscle regeneration. Indeed, a heightened inflammatory response has previously been reported in the skeletal muscle of elderly individuals in response to modest muscle damage (Thalacker-Mercer, Dell'Italia et al. 2010), which is associated with altered impaired regenerative capacity (Merritt, Stec et al. 2013).

Following 12-weeks of regular exercise however, an attenuated response in the inflammatory factors was observed indicating an adaptation to regular exercise. A similar attenuated response of inflammatory factors to acute exercise following a period of training in CKD participants has been reported in previous work by our group (Watson, Viana et al. 2017), and in elderly participants (Della Gatta, Garnham et al. 2014) where it is termed the repeated bout effect (McHugh, 2003). However, it is unknown if this is due to a reduction in immue cell activation following exercise or if this simply reflects a reduction in mechanical disruption to the muscle fibres occurring following repeated bouts of exercise (McHugh, 2003). If indeed the large increase in inflammatory gene expression observed following the initial bout of unaccustomed exercise in the present study does represent a heightened inflammatory response to modest exercise, the attenuated response following 12-weeks of regular exercise might indicate a positive adaptation has occurred. In contrast, given the apparent importance of inflammatory and immune cells in muscle repair and regeneration an attenuated inflammatory response following regular exercise may represent a diminished ability to further adapt to the current exercise stimuli (i.e. intensity), which may therefore need to be increased.

Unfortunatley, the aforementioned limitations having of a singe biopsy time point of 24h post exercise, and the lack of a second baseline biopsy preclude any conclusions being drawn regarding changes in the gene expression of inflammatory factors in response to AE or CE. Therefore future research is needed to investigate the significance of both increasing the expression inflammatory factors in an already elevated pro-inflammatory

environment in the skeletal muscle of CKD participants, and the adpatations to chronic exercise training

7.6 Limitations

Whilst control and CKD participants were well matched for age and sex, differences between the groups for factors such as physical activity levels, presence of co-morbidities, medication, and body composition may have potentially confounded this comparison. Moreover, whilst no systematic differences existed between the expression of 18S between CKD and control samples suggesting it was a suitable housekeeping gene, differences in the way biopsies were collected and stored (i.e. in liquid nitrogen vs. -80°C) may also have confounded the results present in this chapter. Furthermore, as in the previous chapter, the biopsy sampling timpoints for partcipants perfroming exercise and the lack of a healthy control group completing the same exercise protocol limits the conclusions that can be made from the data presented in this chapter.

Methodological limitations also limit the conclusions that can be drawn from the above data. Whilst the cellular adaptations to exercise training are due to the cumulative effects of transient increases in gene transcription (Hargreaves, Cameron-Smith 2002), the absence of a measure of protein content means it is not possible to know if the changes in mRNA expression reported above translate to changes in protein expression. In line with this, a direct measurement of satellite cell number was not performed in the present analysis. For the first few patients to undergo the muscle biopsy a small portion of tissue was embedded in Tissue-Tek O.C.T with the intention of performing immunostaining for cell numbers and expression of the MRFs. However, although the microbiopsy method of muscle biopsy has been validated against the more traditional Bergström technique (Hayot, Michaud et al. 2005), when cross sections were cut, there were not a sufficient number of fibres to perform analysis (Mackey, Kjaer et al. 2009).

7.7 Conclusions

In conclusion, this chapter reports for the first time increased expression of myostatin, its receptor, ActIIBR, and pro-inflammatory cytokines in the skeletal muscle of CKD patients in the earlier stages of disease. In contrast to previous reports in animal models, gene expression of the myogenic regulators were not lower in the muscle of CKD participants in comparison to age- and sex-matched controls. However, only minimal change in the expression of the MRFs was observed following unaccustomed AE or CE, which may indicate impairments in satellite cell activation, proliferation and differentiation in the untrained state. Moreover, the failure to upregulate the MRFs occurred despite the suppression of myostatin and increased expression of pro-inflammatory cytokines, both of which have the potential to stimulate satellite cell activation. Further research is required to investigate the time course of SC activation following exercise in non-dialysis CKD patients.

Chapter 8

General Discussion

The primary aim of this thesis was to investigate the effects of performing supervised aerobic or combined aerobic and resistance exercise on skeletal muscle hypertrophy, physical function, and the molecular responses in patients with CKD stage 3b-5 not requiring dialysis. The primary hypothesis tested was that combined aerobic and resistance exercise would result in greater muscle hypertrophy and increases in muscle function in comparison to aerobic exercise alone (**Chapter 5**), which would occur with altered expression of genes and proteins known to be associated with the regulation of muscle mass in CKD (**Chapters 6, and 7**). Sub analyses are also presented investigating the associations between muscle atrophy and physical performance in the same cohort of CKD participants (**Chapter 4**).

8.1 Summary of key findings

8.1.1 Muscle atrophy is independently associated with physical performance in CKD.

Muscle wasting and dysfunction are commonly reported in CKD populations and are likely to impact upon physical performance. **Chapter 4** reports that the presence of low muscle mass defined using BIA derived ALMI cut-offs is independently associated with lower extremity strength in non-dialysis CKD populations. Moreover, rectus femoris anatomical CSA obtained using skeletal muscle ultrasound was also independently associated with knee extensor strength, and VO_{2peak}. Although, the cross-sectional nature of this chapter precludes the identification of any causal relationships, these results suggest that muscle wasting that starts early in the disease may affect physical performance in non-dialysis CKD patients. Given the important associations between muscle wasting and reduced lower extremity physical performance with important clinical outcomes (Harada, Suzuki et al. 2017, Roshanravan, Robinson-Cohen et al. 2013, Sietsema, Amato et al. 2004), these findings highlight the need for interventions that are capable of improving both muscle mass and function in this population.

8.1.2 Combined aerobic and resistance exercise results in greater improvements in muscle hypertrophy and strength than aerobic exercise alone.

Both aerobic and resistance exercise elicit important benefits for CKD patients when performed alone, therefore performing a combination of both is frequently advocated (Heiwe, Jacobson 2011, Smart, Williams et al. 2013). However, no studies have investigated the efficacy of combining aerobic exercise with progressive resistance exercise in non-dialysis CKD.

Chapter 5 reports the effects of a 12-week exercise intervention consisting of aerobic exercise alone or combined aerobic and resistance exercise in non-dialysis CKD patients. Both 12-weeks of aerobic or combined exercise increased quadriceps volume assessed by MRI and knee extensor strength. Very few studies have reported the impact of aerobic exercise on muscle hypertrophy, but those that have report conflicting results (Baria, Kamimura et al. 2014, Kosmadakis, John et al. 2012). However, the results presented in **Chapter 5** suggest that aerobic exercise is capable of eliciting muscle hypertrophy in non-dialysis CKD, albeit to a lesser extent than when combined with resistance exercise. The improvements in muscle size and strength were greater in those performing combined aerobic and resistance exercise, showing increases that were double that of the aerobic only group. The percentage increase in muscle volume of 9.4% following combined aerobic and resistance exercise is in line with previous reports of resistance exercise interventions in non-dialysis CKD (Watson, Greening et al. 2014).

8.1.3 No change in intramuscular amino acid concentrations following aerobic or combined exercise.

Previous work form our group identified depletion of intramuscular amino acids following aerobic exercise, which may compromise patients' ability to increase muscle mass (Watson, Kosmadakis et al. 2013). The results presented in **Chapter 6** do not support this concept, with no changes in intramuscular BCAA concentrations observed following aerobic or combined exercise. This may be reflected in the increased

quadriceps muscle volume following 12-weeks of either aerobic or combined exercise reported in **Chapter 5**.However, whilst the aforementioned study by Watson and colleagues (2013) reported no change in muscle mass following aerobic exercise that occurred with the depletion of intrmusclular BCAA concentrations, it is likely that methodological differences in the method of muscle mass measurement may account for the discrepent findings. Taken together these results suggest that the amino acid transport mechanisms responsible for transport of amino acids into the muscle cell were not inhibited by AE or CE. However, without a measurement of the expression or activity of the transporters involved conclusions cannot be drawn regarding this.

8.1.4 Impairments in intracellular signalling are overcome following 12-weeks of combined aerobic and resistance exercise in non-dialysis CKD.

Chapter 6 also investigated the signalling responses following unaccustomed and accustomed exercise. Both aerobic and resistance exercise performed alone or in combination have been shown to increase IGF-1 signalling in animal models of CKD and haemodialysis patients. Moreover, in healthy individuals, exercise, and more prominently resistance exercise is known to increase muscle protein synthesis and the associated signalling proteins for up to 48 hours following a single bout of exercise (Phillips, Tipton et al. 1997). However, **Chapter 6** reported that both unaccustomed aerobic and combined exercise failed to increase p-Akt in the skeletal muscle patients with non-dialysis CKD, however, this appeared to be restored following 12-weeks of regular CE where MBI revealed the increase in p-Akt was *likely* greater than response of the AE only group. This further highlights the importance of incorporating resistance exercise in exercise programmes aiming to combat muscle wasting in non-dialysis CKD. However, the fact that muscle hypertrophy observed in **Chapter 5** occurred with both aerobic and combined exercise suggests that both exercise modes increased muscle protein synthesis.

Muscle hypertrophy in response to exercise is predominetly thought to occur through mTORC1 activation that occurs independent of increased IGF-1/Akt signalling (Hamilton, Philp et al. 2010, Philp, Hamilton et al. 2011). Moreover, in addition to Akt

independent activation of mTORC1, recent data suggest that other mechanisms are capable increasing protein synthesis following acute exercise. Indeed, Philp and colleagues (2015) usied rapamycin to inhibit mTORC1 activity following endurance exercise, however this did not blunt myofibrillar and mitochondrial protein synthesis, suggesting other mechanisms are cabale of stimulating protein synthesis following exercise. Therefore it is possible that in the present thesis both AE and CE activated signalling pathways independent of IGF-1/p-Akt leading to increased protein synthesis and ultimately muscle hypertrophy.

8.1.5 Impaired myogenic regulatory factor response to aerobic or combined exercise in CKD

Animal models of CKD exhibit impaired satellite cell dysfunction with reduced expression of the MRFs, which are overcome following overloading (Zhang, Rajan et al. 2011, Wang, Du et al. 2009). In contrast, in **Chapter 7** no differences were observed between the gene expression of the MRFs in the skeletal muscle of CKD participants with age- and sex-matched healthy controls. Moreover, **Chapter 7** also reports possible impairments in satellite cell activation and proliferation in response to both aerobic and combined exercise in CKD participants. Notably MyoD, which controls satellite cell proliferation, was reduced by a mean of 71% below baseline levels following unaccustomed CE, which was deemed *likely decreased* in comparison to AE. However, a mean increase in MyoD above baseline levels was observed following 12-weeks of regular CE. The difference between the unaccustomed and accustomed response following CE was deemed *likely* increased above the AE only group, which may indicate partial restoration following 12-weeks of CE. It is possible that the impairments in satellite cell function following exercise observed here are associated with the impaired IGF-1/Akt signalling following exercise reported in **Chapter 6.** Similar impairments in satellite cell activation and proliferation have been observed in animal models of CKD that occurred as a result of suppressed p-Akt signalling, resulting in the reduced expression of the MRFs (Zhang, Wang et al. 2010).

Chapter 7 also reports increased gene expression of myostatin and ActIIBR gene expression in the skeletal muscle of CKD participants in comparison with healthy controls. However, both AE and CE appear to be effective at suppressing the gene expression of myostatin following acute exercise. Whilst myostatin negatively regulates satellite cell activation (McCroskery, Thomas et al. 2003) the reduction in myostatin gene expression was not associated with an upregulation of the MRFs. However, suppression of myostatin in CKD models results in the reversal of muscle wasting (Zhang, Rajan et al. 2011) and is associated with beneficial effects in other muscle wasting pathologies (Dong, Dong et al. 2017, Zhang, Rajan et al. 2011), therefore further investigation is warranted to determine the effects of myostatin suppression in patients with CKD.

8.1.6 Elevated intramuscular expression of inflammatory cytokines following unaccustomed exercise

Chronic inflammation is frequently associated with muscle wasting in CKD. In this cohort of non-dialysis CKD participants, baseline levels of TNF-α and IL-6 were elevated above healthy control participants by greater than 5-fold (**Chapter 7**). Previous studies have demonstrated elevated levels of TNF-α and IL-6 in the skeletal muscle of CKD patients undergoing surgery to insert a peritoneal catheter, and those receiving dialysis (Garibotto, Sofia et al. 2006, Verzola, Procopio et al. 2011, Zhang, Pan et al. 2013), however this is the first report of elevated inflammatory factors in the skeletal muscle in the earlier stages of CKD. Moreover, following unaccustomed AE and CE substantial increases in the mRNA expression of TNF-α, MCP-1, and IL-6 were observed. However, this response was not observed following 12-weeks of either AE or CE, suggesting an adaptive mechanism to regular exercise. Whilst it is hypothesised that the pro-inflammatory response following unaccustomed exercise represents an immune response to muscle damage, the cumulative effects added to an already pro-inflammatory state are unknown, therefore further work is required to investigate the inflammatory and immune responses to exercise in CKD patients.

8.2 Unanswered questions

8.2.1 Is there an abnormal response to unaccustomed exercise in CKD?

Chapter 6 reported possible impairments in IGF-1/Akt signalling in response to unaccustomed exercise, which confirms the results of previous work by our group that identified the same impaired response following unaccustomed resistance exercise (Watson, Viana et al. 2017). When taken together this suggests a consistent abnormality in IGF-1/Akt signalling following unaccustomed exercise, that given the important role Akt plays in the control of muscle protein metabolism in CKD, warrants further investigation to determine potential underlying mechanisms. Furthermore, if patients exhibit a similar limited response to other anabolic stimuli such as feeding, it may provide an insight into why CKD patients experience muscle wasting. Given the discrepancies between changes in signalling between phosphorylation of anabolic factors, protein synthetic rates and phenotypical adaptations (Greenhaff, Karagounis et al. 2008) future studies should incorporate a direct measurement of muscle protein turnover utilising the incorporation of stable isotopes or doubly labelled water into the muscle tissue. Moreover, a direct comparison between the protein synthetic response to exercise in CKD and well-matched healthy control participants will allow for the identification of any disease specific impairments in muscle protein metabolism.

In addition to impaired IGF-1/Akt signalling, the possible abnormal response of the MRFs in following acute exercise reported in **Chapter 7** requires further investigation. Again, previous work by our group using identical biopsy sampling also reported a similar failure to upregulate the MRFs at 24h following unaccustomed and accustomed exercise (Watson, Viana et al. 2017). It is currently unclear if this represents a failure to respond to an exercise stimulus, or if the time course of these MRFs is altered in CKD. Future research should investigate the response of the MRFs at a number of time points following exercise. Moreover, in addition to examining gene expression of regulatory factors, future work should also aim to determine the total number of satellite cells, and the percentage expressing the MRFs using techniques such as immunohistochemistry and/or flow cytometry.

8.2.2 What are the effects of exercise on mitochondrial abnormalities in CKD?

Further to the mechanisms described in this thesis resulting in the loss of muscle proteins, patients with CKD also exhibit mitochondrial dysfunction (Gamboa, Billings et al. 2016, Su, Klein et al. 2017), which contributes to muscle wasting and dysfunction. Future analysis of the biopsies collected from the ExTra CKD study will investigate the effects of aerobic or combined exercise on mitochondrial enzyme activity and capacity for oxidative phosphorylation.

8.3 Limitations

Potential limitations of the work presented in the individual chapters are discussed within the appropriate discussion sections, however there are a few general limitations that need to be taken into account when viewing the data presented in this thesis as a whole.

Firstly, sample size calculations were not performed for all of the individual analyses and specific aims within the individual chapters. For the cross-sectional analyses in **Chapter 4** the main aim was to describe the cohort taking part in the ExTra CKD study in terms of sarcopenia prevalence and physical performance, therefore no power calculation was performed, and the findings should be confirmed in an appropriately powered longitudinal study. The primary aim of the ExTra CKD study presented in **Chapter 5** was to generate samples (muscle biopsy and blood) to extend the previous work by our group, and as such was powered based on participants achieving a training load sufficient to elicit a similar detectable physiological response following exercise as previously reported (Watson, Greening et al. 2014). However, this means that the study was not powered to detect differences within or between groups for the physiological outcomes presented in **Chapter 5**. Similarly, as there was little data regarding the cellular responses to different modes of exercise in CKD populations, the changes in gene and protein expression presented in **Chapters 6 and 7** were not powered to detect statistical differences within or between groups.

Secondly, the inclusion criteria for participants included in the ExTra CKD study (presented in Chapter 3) meant that not all participants were muscle wasted/sarcopenic. Indeed, the prevalence of sarcopenia of participants enrolled in the ExTra CKD study reported in **Chapter 4** ranged between 16-34%. Therefore this thesis cannot draw conclusions regarding the physiological or cellular responses to aerobic or combined exercise in muscle wasted participants with CKD. This should be investigated in appropriately designed future studies. Moreover, in line with this, the demanding nature of exercise research means that there may have been a selection bias towards 'healthier' participants and those who had more time to dedicate to the research (i.e. retired individuals).

Finally, the lack of resistance exercise only group means that conclusions cannot be drawn regarding any interference between aerobic and resistance exercise modes when combined. Whilst the muscle hypertrophy observed in **Chapter 5** following combined exercise is similar to that previously reported following resistance exercise only in non-dialysis CKD patients (Watson, Greening et al. 2014) the duration of the interventions were different, and therefore a direct comparisons cannot be made.

8.4 Wider clinical implications and future directions

8.4.1 Importance of increasing muscle mass and physical function in CKD

As discussed in **Chapter 1** strong associations between surrogates of muscle mass and mortality are observed in non-dialysis CKD patients (Pereira, Cordeiro et al. 2015, Harada, Suzuki et al. 2017, Chang, Wu et al. 2011). Moreover, as demonstrated in **Chapter 4**, low muscle mass is associated with reduced physical functioning, which is likely to negatively impact other aspects of patients' lives. Indeed, physical impairment is a critical link between muscle dysfunction and disability, defined as difficulty or an inability to perform activities of daily living (Roshanravan, Gamboa et al. 2017). This is of particular importance as physical dysfunction and disability are likely to contribute to unemployment, reduced ability to perform activities of daily living (ADL) and self-care

and/or care for others, loss of independence, and ultimately reduced QoL. All of which are common in CKD populations.

Moreover, physical dysfunction likely contributes to poor metabolic and cardiovascular health in CKD through reduced physical activity levels. Indeed, muscle wasting is an important pathophysiological determinant of physical activity in CKD (Zelle, Klaassen et al. 2017). Moreover, low muscle mass has been shown to associate with low physical activity levels in individuals with reduced kidney function (Baxmann, Ahmed et al. 2008). Whilst physical inactivity likely contributes to muscle wasting, low muscle mass contributes to a cycle of deconditioning that includes reduced physical performance, and physical inactivity (Figure 8.1), which ultimately results in increased cardiovascular risk in CKD populations (Kosmadakis, Bevington et al. 2010)

Importantly, the data presented in **Chapter 5** suggests that muscle wasting and dysfunction are modifiable through appropriate exercise and offer important targets to reverse the downward spiral of deconditioning. Therefore it is plausible to speculate that improvements in muscle mass and physical performance may beneficially impact upon on the factors described above, including physical activity levels, and therefore cardiovascular risk, which is the number one cause of mortality amongst CKD patients. Moreover, higher levels of habitual physical activity are associated with a slower decline in kidney function (Robinson-Cohen, Katz et al. 2009, Robinson-Cohen, Littman et al. 2014) therefore by increasing physical performance and physical activity levels in CKD, it might be possible to slow the deterioration in kidney function.

However, whilst this thesis has demonstrated beneficial short-term effects of performing 12-weeks of either aerobic or combined exercise on quadriceps muscle mass and function; the long-term effects are currently unknown. Future studies should aim to investigate the long-term effects of increasing quadriceps muscle mass and strength in non-dialysis CKD patients, and whether improvements are associated with better prognosis.

8.4.2 Heath care costs and utilisation

Muscle wasting is also associated with increased healthcare costs. Loss of muscle mass and physical function are characteristics of frailty syndrome which is common in both dialysis dependent and independent CKD patients, and is associated with increased risk of falls, health care usage and costs (Chowdhury, Peel et al. 2017, Drost, Kalf et al. 2016, Musso, Jauregui et al. 2015). Reduced muscle strength and frailty are important risk factors for falls which cost the NHS over £2 billion a year, and is forecast to increase (Public Health England 2014). Indeed, the presence of frailty is an important risk factor of accidental falls in CKD populations (Lopez-Soto, De Giorgi et al. 2015) where the cumulative 1-year mortality rates following serious falls is far greater than that of age matched controls (21% vs. 5.5%) (Bowling, Bromfield et al. 2016)

Moreover, the presence of cachexia in a number of disease states is also associated with increased healthcare usage. Using data from the United States Nationwide Inpatient Sample, Arthur et al (2014) reported that the duration of hospital stay was longer in patients with cachexia, with the median stay being double that of individuals without cachexia (median 6 vs. 3 days,) corresponding to an increased average cost of $4641 per hospital stay. Therefore it is plausible to speculate that performing appropriate exercise incorporating resistance exercise (as demonstrated in this thesis) to improve lower limb muscle mass and function in non-dialysis CKD may attenuate the development of frailty/cachexia syndromes that are associated with increased healthcare costs. However, this needs to be determined in appropriately designed longitudinal studies.

Figure 8.1 Cycle of muscle wasting, physical dysfunction and physical inactivity in CKD. As described in previous chapters, reduced kidney function is associated with a catabolic state, resulting in muscle wasting and dysfunction. This in turn impacts physical function, which interferes with physical activity levels, leading to reduced anabolic stimuli to the muscle, and further compromising muscle mass. Reduced physical function and activity is also associated with increased risk of mortality in CKD populations.

8.4.3   Recommendations for guidelines

Currently, cohesive guidelines for the provision of exercise in CKD do not exist. Whilst exercise has recently been incorporated into national and international guidelines for the treatment and management of CKD (National Institute for Health and Clinical Excellence 2014, K/DOQI Clinical Practice Guidelines for Cardiovascular Disease in Dialysis Patients. 2005, KIDGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. 2013), they lack detail regarding the, frequency, intensity, type, and duration of exercise. More comprehensive guidelines have proposed a combination of aerobic and resistance exercise should be undertaken, however this recommendation has largely been based on evidence from studies investigating the effects

of aerobic or resistance exercise performed separately (Smart, Williams et al. 2013, Heiwe, Jacobson 2011). The work presented in **Chapter 5** of this thesis provides evidence for the efficacy of performing combined aerobic and resistance exercise over aerobic exercise alone, whilst **Chapter 6 and 7** highlight the importance of the addition of resistance exercise at modulating the pathways that result in the loss of muscle mass in CKD populations. Taken together this data provides evidence to inform the recommendations of combined aerobic and resistance exercise over performing aerobic exercise alone in non-dialysis CKD, and will help inform future exercise guidelines in this population.

8.5 Concluding remarks

Skeletal muscle wasting and dysfunction are common in CKD, and closely associate with reduced physical performance and mortality. This thesis has demonstrated that 12-weeks of combined aerobic and resistance exercise results in superior muscle hypertrophy and strength improvements in comparison to performing aerobic exercise alone. This may have occurred with the modulation of key molecular pathways that have been implicated in CKD induced muscle wasting. However, both exercise modes had similar effects on physical performance outcomes, which need to be investigated further in appropriately designed trials. Taken together the work presented in this thesis demonstrates the importance of incorporating resistance exercise in programmes aiming to increase muscle size and strength in non-dialysis CKD, and suggests that it can be combined effectively with aerobic exercise to produce superior benefits in terms of increasing muscle size and strength compared to performing aerobic exercise alone.

Appendices

Appendix A – Solutions & Reagents

Western Blot Buffers

Laemmli reducing sample buffer

4ml ultra pure H_2O

1ml 0.5M Tris HCL pH 6.8

0.8ml Glycerol

1.6ml 10% w/v Sodium Dodecyl Sulphate (SDS)

0.4ml β-Mercaptoethanol

0.2ml 0.05% w/v Bromophenol Blue

10X Running Buffer (1 litre)

30.0g Trizma Base

144g Glycine

100ml 20% SDS

Ultra pure water to 1 litre

1X Running Buffer (1 litre)

100ml 10X running buffer

900ml ultra pure water

10X Transfer Buffer (1 litre)

30.3g Trizma Base

144g Glycine

Ultra pure water to 1 litre

1X Transfer Buffer (1 litre)

100ml 10X transfer buffer

100ml Methanol

800ml ultrapure water

10X TBS (1 litre)

60.55g Trizma base

87.66g NaCl

~700ml ultra pure water

pH to 7.6 using 6M HCL

Ultra pure water to 1 litre

1X TTBS

100ml 10X TBS

1ml Tween 20

Ultrapure water to 1 litre

Casting gels

12.5% SDS Gel (2 gels)

Resolving gel

4.79ml ultra pure water

6.26ml Acrylamide

7.5ml Tris (pH8.8)

300μl SDS (10%)

150μl APS (10%)

15μl TEMED

Stacking gel

9.08ml ultrapure water

1.95ml acrylamide

3.75ml Tris (pH 6.8)

150μl SDS (10%)

75μl APS (10%)

15μl TEMED

10% SDS gel (2 gels)

Resolving gel

6.2ml ultrapure water

5ml acrylamide

3.75ml Tris (pH 8.8)

150μl SDS (10%)

75μl APS (10%)

20μl TEMED

Stacking gel

4.6ml ultrapure water

1ml acrylamide

1.9ml Tris (pH 6.8)

75μl SDS (10%)

37.5μl APS (10%)

11μl TEMED

6% SDS gel (2 gels)

Resolving gel

8ml ultrapure water

3.75ml Tris (pH 8.8)

3ml acrylamide

150μl SDS (10%)

75μl APS (10%)

20μl TEMED

Stacking gel

4.60ml ultrapure water

1ml acrylamide

1.9ml Tris (pH 6.8)

75μl SDS (10%)

37.5μl APS (10%)

11μl TEMED

Folin Lowry Assay Reagents

Reagent A (1 litre):

20g Anhydrous Na_2CO_3

4g NaOH

0.2g K, Na-tartrate

Ultra pure water up to one litre

Reagent B (1 litre)

5g $CuSO_4 5H_2O$

Ultra pure water to 1 litre

Sodium dihydrogen solution for HPLC

1 liter:

6.24g sodium dihydrogen

~700ml ultrapure water

pH to 7.8 using 47% v/v NaOH

ultrapure water to 1litre & filtred

Appendix B – Amino acid standard & concentrations

Amino Acid	Approx. Elution Time (min)	Final Concentration (uM or pmol/ul)
L-Aspartate	0.97	248
L-Glutamate	2.01	248
L-Asparagine*	3.56	256
L-Serine	3.70	248
L-Glutamine*	4.06	253
L-Histidine	4.19	248
Glycine	4.37	248
L-Threonine	4.48	248
L-Arginine	4.79	248
L-Alanine	5.14	248
L-Tyrosine	5.76	248
L-Cystine	6.55	248
L-Valine	6.80	248
L-Methionine	6.92	248
L-Norvaline*	7.11	248
L-Tryptophan*	7.38	248
L-Phenylalanine	7.60	248
L-Isoleucine	7.71	248
L-Leucine	8.06	248
L-Lysine	8.28	248

Muscle amino acid concentration (mmol/l cell H$_2$O) in the fasted state following at baseline, following unaccustomed exercise, and accustomed AE and CE. Data presented as mean ± SD

Amino Acid	Aerobic			Combined		
	Baseline	Untrained	Trained	Baseline	Untrained	Trained
L-Glutamate	8.1 ± 6.0	7.7 ± 5.9	6.8 ± 6.5	2.8 ± 4.6	2.7 ± 4.4	2.8 ± 4.8
L-Asparagine*	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
L-Serine	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.2	0.3 ± 0.1	0.4 ± 0.2	0.3 ± 0.1
L-Glutamine*	15.5 ± 3.1	15.1 ± 5.1	15.8 ± 6.1	16.0 ± 11	17.5 ± 10.3	18.5 ± 15.6
L-Histidine	0.6 ± 1.0	0.3 ± 0.6	0.2 ± 0.5	0.3 ± 0.3	0.1 ± 0.1	0.02 ± 0.1
Glycine	2.4 ± 2.2	2.2 ± 2.0	2.3 ± 1.8	3.4 ± 6.7	3.4 ± 6.5	3.1 ± 6.2
L-Threonine	0.4± 0.3	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.4	0.4 ± 0.5	0.4 ± 0.4
L-Arginine	0.7 ± 1.1	0.9 ± 1.2	1.0 ± 1.8	0.4 ± 0.4	0.3 ± 0.3	0.3 ± 0.3
L-Alanine	1.9 ± 1.3	1.6 ± 0.8	1.5 ± 0.9	2.3 ± 1.7	2.1 ± 1.7	2.3 ± 2.0
L-Tyrosine	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2
L-Cystine	0.2 ±0.2	0.1 ± 0.2	0.1 ± 0.1	1.9 ± 4.5	1.0 ± 2.2	2.0 ± 5.2
L-Valine	0.3 ± 0.3	0.3 ± 0.2	0.5 ± 0.4	0.4 ± 0.3	0.4 ± 0.2	0.3 ± 0.2
L-Methionine	0.2 ± 0.3	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.03 ± 0.1	0.01 ± 0.03
L-Tryptophan*	-	-	-	-	-	-
L-Phenylalanine	0.3 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1

L-Isoleucine	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.4 ± 0.9
L-Leucine	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
L-Lysine	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.3	0.5 ± 0.4	0.3 ± 0.3	0.3 ± 0.3

Plasma amino acid concentration (mmol/l cell H₂O) in the fasted state following at baseline, following unaccustomed exercise, and accustomed AE and CE. Data presented as mean ± SD

Amino Acid	Aerobic			Combined		
	Baseline	Untrained	Trained	Baseline	Untrained	Trained
L-Glutamate	143.1 ± 107.2	144.7 ± 95.7	138.7 ± 110.7	123.9 ± 108.9	132.7 ± 110.8	113.7 ± 111.4
L-Asparagine*	44.6 ± 6.1	45.5 ± 22.5	48.6 ± 12.4	46.8 ± 10.5	48.3 ± 11.7	46.7 ± 8.7
L-Serine	93.5 ± 31.4	97.0 ± 33.0	100.0 ± 33.1	85.2 ± 15.3	92.4 ± 19.1	87.0 ± 17.8
L-Glutamine*	578.4 ± 92.0	603.9 ± 152.5	576.1 ± 143.6	583.5 ± 123.9	593.6 ± 112.8	581.5 ± 112.8
L-Histidine	51.1 ± 13.6	58.7 ± 18.7	69.4 ± 25.2	53.0 ± 6.9	54.5 ± 18.4	57.7 ± 21.1
Glycine	262.4 ± 63.7	286.0 ± 65.1	288.7 ± 57.1	367.8 ± 171.1	363.2 ± 106.3	332.9 ± 106.3
L-Threonine	110.7 ± 39.4	124.4 ± 51.8	118.6 ± 50.5	102.4 ± 23.3	94.7 ± 31.4	96.5 ± 30.8
L-Arginine	102.8 ± 31.9	95.4 ± 40.9	97.2 ± 29.8	118.3 ± 40.3	95.9 ± 36.4	92.7 ± 28.0
L-Alanine	486.1 ± 108.1	547.5 ± 194.7	529.0 ± 148.0	498.1 ± 178.2	501.6 ± 137.2	462.1 ± 85.4
L-Tyrosine	58.0 ± 21.4	62.4 ± 32.6	58.3 ± 22.2	59.2 ± 23.3	58.7 ± 21.1	57.5 ± 23.3
L-Cystine	78.4 ± 40.8	85.6 ± 34.5	81.6 ± 32.8	75.4 ± 20.5	78.8 ± 25.5	76.6 ± 29.1
L-Valine	250.1 ± 71.4	247.1 ± 112.7	241.9 ± 94.6	209.8 ± 68.6	193.2 ± 63.3	194.7 ± 71.8
L-Methionine	23.7 ± 5.3	24.0 ± 5.3	23.3 ± 7.9	20.0 ± 6.1	18.8 ± 6.9	18.4 ± 4.6
L-Tryptophan*	-	-	-	-	-	-
L-Phenylalanine	61.5 ± 12.1	63.6 ± 19.6	59.0 ± 17.0	64.4 ± 21.2	72.3 ± 36.4	64.1 ± 17.6

L-Isoleucine	69.5 ± 22.9	76.0 ± 30.1	73.7 ± 27.8	95.4 ± 73.2	88.6 ± 62.3	102.8 ± 123.7
L-Leucine	131.6 ± 33.9	132.4 ± 58.0	135.6 ± 55.0	147.0 ± 57.1	137.8 ± 57.4	141.8 ± 76.8
L-Lysine	196.7 ± 73.5	184.6 ± 92.8	209.4 ± 89.5	174.4 ± 75.8	177.7 ± 81.4	168.8 ± 52.4

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