Evaluation of 11β-hydroxysteroid dehydrogenase type 1 as a therapeutic target to treat sarcopenia in chronic kidney disease

By

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Abstract

The decline of muscle mass and strength, termed sarcopenia, is common in people with chronic kidney disease (CKD). Even though coexisting sarcopenia profoundly increases mortality and morbidity in CKD, no targeted treatments to correct the underlying disturbances in skeletal muscle metabolism are currently available.

This PhD thesis examines the hypothesis that excessive peripheral production of the hormone cortisol within skeletal muscle tissue by the enzyme 11beta hydroxysteroid dehydrogenase type 1 (11βHSD1) contributes to loss of muscle mass and strength in CKD, and that inhibition of 11βHSD1 has potential as a novel therapy to mitigate sarcopenia in CKD.

The scientific approach to explore this hypothesis comprises a range of preclinical and clinical research methodologies. The role of 11βHSD1 for muscle atrophy and consequences of its suppression are evaluated in vivo using a mouse model of persistent renal impairment. Complementary in vitro experiments using human muscle cells are conducted to elucidate regulation of 11βHSD1 by factors associated with CKD. Finally, 11βHSD1 activity is quantified directly in skeletal muscle biopsies in a case-control study involving volunteers with and without CKD to detect associations with kidney disease and muscle phenotype.

The data from independent preclinical and clinical lines of investigations indicate that 11βHSD1 activity in skeletal muscle tissue in conditions of CKD is unchanged compared to control conditions. Interestingly, the observational data from humans reveals that muscle 11βHSD1 activity is positively correlated with age and negatively correlated with muscle performance

markers (grip strength, gait speed), regardless of CKD status. Nevertheless, genetic silencing of 11βHSD1 did not alter the muscle atrophy phenotype in mice with renal impairment. Compensatory elevation of circulating glucocorticoid levels is observed in mice without 11βHSD1 function, which may undermine the therapeutic efficacy of 11βHSD1 suppression.

In summary, the evidence gathered as part of this PhD thesis does not support significant upregulation of 11βHSD1 in skeletal muscle in response to reduction of kidney function or a major contribution to CKD-related sarcopenia risk. The upregulation of 11βHSD1 activity in skeletal muscle with age is consistent with previous reports and poses the question of its functional significance for age-related changes in skeletal muscle. The thesis concludes with a discussion of potential therapeutic niches for 11βHSD1 inhibitors that remain for future exploration.

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Contents

List of Figures

List of Tables

List of Abbreviations

11βHSD	11beta-hydroxysteroid dehydrogenase
11βHSD1-KO/11βKO	11beta-hydroxysteroid dehydrogenase type 1 – gene knock-out
2,8-DHA	2,8 dihydroxyadenine
4E-BP1	eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1
5αTHF	5-alpha-tetrahydrocortisol
ACR	albumin-creatinine-ratio
ACTH	adrenocorticotropic hormone
ActRIIB receptor	activin receptor type-2B
Akt	protein kinase B
ANOVA	analysis of variance
ASM	appendicular skeletal muscle mass
AZD4017	small molecule inhibitor of human 11βHSD1
BMI	body mass index
C.I.	confidence interval
C/EBPβ	CCAAT/enhancer-binding protein beta
C2C12	an immortalised mouse myoblast cell line
C57BL/6	C57 black 6 mouse strain
CBG	cortisol binding globulin
cDNA	complementary DNA
CKD	chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CO2	carbon dioxide
COPD	chronic obstructive pulmonary disease
CPM	counts per minute
Creat	creatinine
CRH	corticotrophin releasing hormone
CRP	C-reactive protein
CVD	cardiovascular disease
cysC	cystatin C

DAPI	4’,6-diamidino-2-phenylindole
DEXA	Dual-energy X-ray absorptiometry
DHC	11-dehydrocorticosterone
DHEAS	dehydroepiandrosterone sulphate
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
eGFR	estimated glomerular filtration rate
ELISA	enzyme-linked immunosorbent assay
EQ-5D	name of a research instrument (not an acronym)
EWGSOP	European Working Group on Sarcopenia in Older People
F10 media	Ham’s F-10 Nutrient Mix
Fbxo32	F-Box Protein 32
FoxO1	Forkhead box protein O1
GA	glycyrrhetinic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	glucocorticoid
Gilz	glucocorticoid-induced leucine zipper
GR	glucocorticoid receptor
H&E	haematoxylin and eosin
H6PD	Hexose-6-Phosphate Dehydrogenase
HbA1c	haemoglobin A1c, a measure of glycated haemoglobin
HC	healthy control
HCl	hydrochloric acid
Hsd11b1	gene encoding 11βHSD1
Hsd11b2	gene encoding 11βHSD2
IGF-1	insulin-like growth factor 1
IL1	interleukin 1
IL6	interleukin 6
IPAQ	international physical activity questionnaire

IRS-1	insulin receptor substrate 1
ISRCTN	International Standard Randomised Controlled Trial Number
JAK/STAT	Janus kinase/signal transducers and activators of transcription
KLF-15	Krüppel-like factor 15
LCMS/MS	liquid chromatography – tandem mass spectrometry
MFX study	muscle-fat crosstalk study
MG132	a potent proteosome inhibitor
miRNA	microRNA
MR	mineralocorticoid receptor
MRI	magnetic resonance imaging
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
Myf5	myogenic factor 5
Myf6	myogenic factor 6
MyoD	myogenic differentiation 1
MyoG	myogenin
NADP+	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NAFLD	non-alcoholic fatty liver disease
NaOH	sodium hydroxide
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
NRES REC	National Research Ethics Service Research Ethics Committee
OR	odds ratio
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK4	pyruvate dehydrogenase kinase 4
PI3K	phosphoinositide 3-kinase
PTEN	phosphatase and tensin homolog
REDD1	regulated in DNA damage and development 1
RIISC study	Renal Impairment in Secondary Care study

RNA	ribonucleic acid
rpm	rotations per minute
RPMI medium	Roswell Park Memorial Institute medium
rtPCR	reverse transcription-polymerase chain reaction
RU-486	mifepristone
SGLT2	sodium-glucose transport protein 2
SMK study	Steroid Metabolism and Muscle Loss in Chronic Kidney Disease study
SOCS3	suppressor of cytokine signalling 3
SPPB	short physical performance battery
THE	tetrahydrocortisone
THF	tetrahydrocortisol
TLC	thin layer chromatography
TNFα	tumour necrosis factor alpha
Trim63	tripartite motif containing 63
UPS	ubiquitin-proteosome system
UV	ultraviolet
WT	wild-type

Chapter I – Introduction

1.1 Background: skeletal muscle & sarcopenia

1.1.1 Skeletal muscle physiological functions

Skeletal muscle typically accounts for one third of human body weight and has wide-ranging physiological functions.[1, 2] Muscles produce movement through converting chemical energy to mechanical force.[3] Movement not only allows locomotion and manipulation of our environment, but is also underpins balance, speech, swallowing, directing visual gaze and breathing. Muscle also performs sensory functions through proprioception. As a major metabolic tissue, muscles are important for storage and homeostatic regulation of carbohydrates and amino acids.[4, 5] Metabolic activity in muscles furthermore produces heat which contributes to maintaining body temperature. Finally, muscle is increasingly recognised not only as a target site for ligands, but also as a tissue that secretes signal mediators termed myokines with autocrine, paracrine and endocrine effects.[6]

1.1.2 Sarcopenia definition

Sarcopenia describes the condition of generalised and accelerated reduction in skeletal muscle mass, strength and function.[7] Muscle mass and strength typically reach a peak in the third decade of life. Noticeable decline in muscle mass and strength occurs from the fifth decade of life onward, with muscle strength declining faster than muscle mass.[8, 9] Even though age is a major risk factor for sarcopenia, the term commonly refers to accelerated or progressive muscle decline beyond changes related to healthy aging alone. As such, sarcopenia can be considered in terms of skeletal muscle insufficiency or failure, characteristically associated with adverse outcomes like falls, fractures, increased care needs and mortality. The condition

may develop rapidly (acute), e.g. in the setting of acute illness or immobilisation, or over a protracted period (chronic).

The term sarcopenia originates from Greek, with *sarx* meaning flesh and *penia* meaning loss, and was first proposed by Irwin Rosenberg in 1988.[10] Recent definitions of sarcopenia place particular emphasis on muscle function, i.e. muscle strength or physical performance, because muscle function has consistently been a stronger predictor of clinically relevant outcomes than muscle mass alone.[11-13] The latest and most widely accepted clinical diagnostic criteria for sarcopenia have been proposed by the European Working Group on Sarcopenia in Older People (EWGSOP) in 2019 [14] (Table 1.1). These criteria distinguish probable sarcopenia (evidence of low muscle strength), confirmed sarcopenia (additional evidence of low muscle quantity or quality) and severe sarcopenia (additional evidence of impaired physical performance).

Table 1.1 EWGSOP2 sarcopenia cut-off points

Test	Cut-offs for men	Cut-offs for women
Cut-off points for low strength		
Grip strength	<27kg	<16kg
Chair stand	>15sec for five rises	
Cut-off points for low muscle quantity		
Appendicular skeletal muscle mass	<20kg	<15kg
Appendicular muscle mass/height^2	<7.0kg/m^2	<5.5kg/m^2
Cut-off points for low performance		
Gait speed	≤0.8m/sec	
Short physical performance battery	≤8 point score	
Time-up-and-go test	≥20sec	
400meter walk test	Non-completion or ≥6min for completion	

Table reproduced with adaptions from [14]

It is worth pointing out that the concept of sarcopenia is distinct from frailty (a syndrome of reduced physiological reserve with reduced resilience to stress events and increased vulnerability to adverse health outcomes) and cachexia (a severe weight loss condition characterised by combined loss of skeletal muscle and adipose tissue), although there is clinical overlap for these conditions and they may coexist in a single person.[14, 15]

1.1.3 Sarcopenia prevalence

Sarcopenia is common in the general population, especially among the elderly. A meta-analysis of 9 studies by Almohaisen et al. estimated sarcopenia prevalence at 14% (95% confidence interval 9-20%) among people aged >50yrs living in the community.[15] Depending on the diagnostic criteria for sarcopenia, another meta-analysis by Petermann-Rocha et al. described a prevalence range between 10% - 27% among adults outside of hospital.[16] Prevalence of sarcopenia is generally higher among populations in care settings such as hospitals or care homes than in community dwelling populations [17, 18], and among populations with chronic health problems.[19]

1.1.4 Sarcopenia principal causes

The underlying causes for sarcopenia are multifactorial, broadly comprising physical inactivity, deficiencies in nutrition and metabolic disturbances in skeletal muscle. Muscle growth and strength development requires physical stimulation through activity and exercise. This is clearly illustrated in studies of voluntary inactivity or forced immobilisation.[20-22] Additionally, adequate nutrition is a prerequisite for maintaining healthy muscle mass and

function. In states of nutrient deficits, especially protein or amino acid deficits, stores in skeletal muscle are broken down and released, promoting muscle atrophy.[23-25] Importantly, the influence of exercise and nutrition depends on metabolic and physiological adaptations in skeletal muscle. Aging and disease not only adversely affect people's diets and capacity for exercise, but also interfere with normal metabolic regulation.[4] While physical inactivity or malnutrition can precipitate sarcopenia, simple lack of physical activity or nutrition does not encapsulate the full pathophysiology of sarcopenia. To understand the root causes of sarcopenia, it is important to draw attention to metabolic disturbances in skeletal muscle.

Regulation of skeletal muscle metabolism and physiology is generally impaired at a fundamental level in sarcopenia. This notion is broadly captured in the concept of "anabolic resistance".[4] Anabolic resistance refers to diminished gains in muscle mass and strength following exercise and nutritional stimuli. It goes hand in hand with a susceptibility to muscle catabolism, atrophy and weakness. There are many underlying reasons for these metabolic disturbances, including inflammation, acidosis, hormonal dysregulation, microvascular changes, oxidative stress or neuronal changes.[7] Given the range of factors interfering with normal metabolism, it is unsurprising that many diseases promote sarcopenia, including heart failure, liver disease, chronic kidney disease, COPD, cancer and others.[19, 26-29] Iatrogenic factors such as certain medications or bed rest may further exacerbate sarcopenia.[23, 30] A more detailed discussion of current knowledge on mechanisms that interfere with normal muscle metabolism and contribute to sarcopenia will follow later with specific relevance to chronic kidney disease (see section 1.4).

1.1.5 Sarcopenia mortality & morbidity

Sarcopenia strongly associates with increased mortality, disability and excess healthcare costs. A recent meta-analysis reported a hazard ratio for mortality of 2.0 (95% confidence interval 1.7-2.3) related to sarcopenia.[31] Higher mortality was evident both among community-dwelling populations and populations in institutional care settings. Another meta-analysis demonstrated an association between sarcopenia and functional disability with an odds ratio of 3.6 (95% confidence interval 3.0-4.4).[32] A wide range of other adverse medical outcomes have been linked with sarcopenia markers and weakness, including risk of falls, fractures, cognitive decline, cardiovascular disease, hospitalisation and reduced quality of life.[32-35] Accordingly, several studies have suggested that sarcopenia is associated with a substantial increase in health and social care expenditure.[36, 37]

1.1.6 Sarcopenia principles of current management

Current treatment approaches to sarcopenia rely largely on non-pharmacological interventions, while pharmacological treatment options are scarce. Physical activity forms a central part of current management guidelines.[38] Substantial evidence supports the benefits of resistance exercise for muscle mass and strength.[39, 40] There has been considerable heterogeneity in terms of the exercise interventions and study populations reported in the literature, with few studies focusing specifically on populations with sarcopenia.[38] A notable exception is the SPRINTT trial, which tested an exercise intervention together with nutritional counselling in older adults with sarcopenia and demonstrated a reduction in the incidence of mobility disability over 36 months.[41] Still, tolerance and motivation represent barriers to

implementing exercise interventions, especially in older adults with comorbidities.[42] Furthermore, the efficacy of exercise interventions is reduced by metabolic dysregulation in people with sarcopenia that cause anabolic resistance in skeletal muscle.[4]

Preventing nutritional deficiencies is important for maintaining healthy muscle mass and strength. Yet, evidence for nutrition interventions to tackle sarcopenia is less consistent, with many studies relying on observational evidence.[7, 38] The benefits of nutritional interventions are likely limited outside the specific setting of sarcopenia with malnutrition.

No medications are available as specific treatments for sarcopenia. Certain studies have suggested beneficial effects of vitamin D or testosterone supplementation in specific subgroups with deficiency for these compounds.[43] Several pharmaceutical approaches such as myostatin antagonists or selective androgen receptor modulators have been tested in Phase I or II without convincing results in larger trials to date.[44, 45]

In summary, the current mainstay for sarcopenia management consists of promoting physical activity, especially resistance-based training. There is a rationale for addressing co-existing nutritional deficiencies, especially low protein intake, but the value of nutritional interventions for universal management of sarcopenia remains controversial. Importantly, lifestyle interventions are challenging to implement in sarcopenic populations and are hampered by anabolic resistance. There are no treatments that address the underlying metabolic disturbances in skeletal muscle that promote sarcopenia. Given the significance of sarcopenia

as a health problem in our aging population, developing novel treatment approaches to correct metabolic dysregulation in skeletal muscle is an urgent research priority.

1.2 Background: the kidneys & chronic kidney disease

1.2.1 Kidney physiological functions

The kidneys have important physiological functions for maintaining homeostasis and excreting metabolic waste products.[46] Receiving 25% of the circulating blood flow, the kidneys control a stable balance for electrolyte concentrations, blood pressure, acid-base status and total body water. The kidneys are also the major organ for excretion of water-soluble waste products or toxins. Finally, the kidneys perform endocrine functions through activation of vitamin D, release of renin and production of erythropoietin.

1.2.2 Chronic kidney disease (CKD) definition

Chronic kidney disease (CKD) describes a syndrome of abnormal kidney structure or function present for three months or more with implications for health.[47] Most commonly, CKD refers to a reduction in glomerular filtration rate below 60ml/min/1.73m^2. CKD is classified according to severity and risk for adverse medical events into five major stages, with stage 1 being least severe and stage 5 being most severe. When kidney function deteriorates to the point that sustaining life is dependent on renal replacement therapy (dialysis or transplantation), it is termed end-stage renal failure.

1.2.3 CKD prevalence

CKD is very common and projected to grow further in prevalence. Estimations of CKD prevalence range between 8-14%.[46, 48] A population health survey in England in 2016 estimated the prevalence for all forms of CKD at 13.9%.[49] Most people with CKD have

moderate impairment of kidney function (estimated prevalence in the US population for CKD stage 3: 7.7%), while some patients have more advanced disease (estimated prevalence in the US population for CKD stage 4-5: 0.5-1%).[50] The number of people living with end-stage renal failure in the UK in 2020 was 68,000, representing 0.13% of the general population, meaning they rely on dialysis or transplantation for survival.[51] Prevalence of CKD increases profoundly with older age (Figure 1.1).[49, 52] While CKD is slightly more common in women than in men [49], men have higher risk of progressing to end-stage renal failure.[51] A modelling exercise by Public Health England estimated 2.6 million people lived in CKD in England in 2011, with a projected rise to 4.2 million people by 2026.[52]

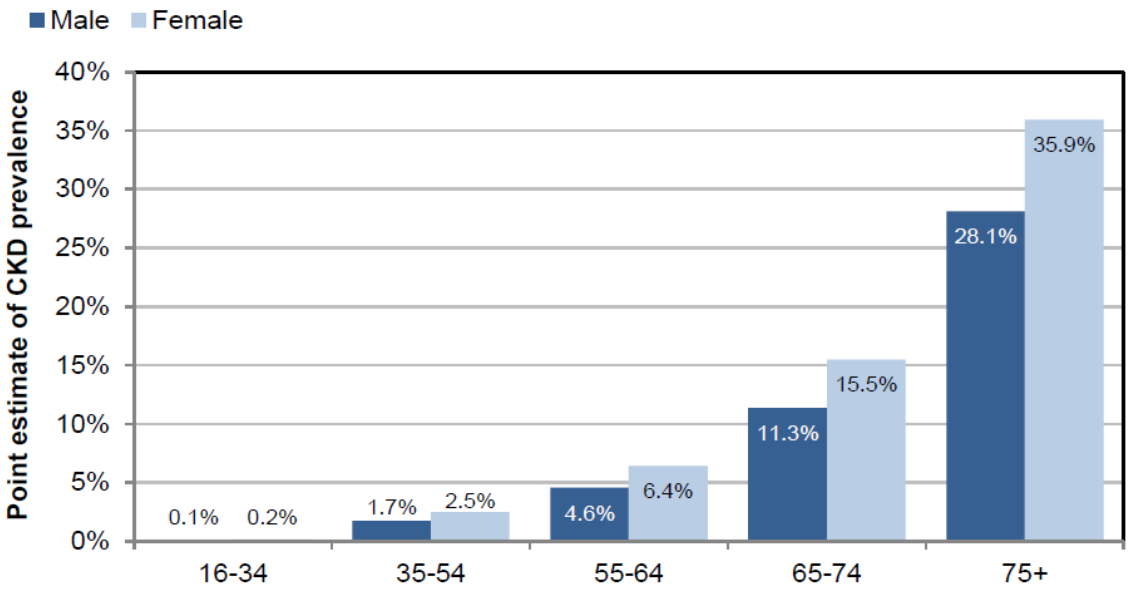


Figure 1.1 Summary of expected CKD stage 3-4 prevalence in England.
Figure reproduced from Barron E. Chronic kidney disease prevalence model. Public Health England, London, UK. 2014. [52]; contains public sector information licensed under the Open Government Licence v2.0.

1.2.4 CKD causes

CKD is caused by a wide range of diseases. The most common cause of CKD in developed countries is diabetes mellitus, accounting for roughly one third of all cases of CKD.[53] About one quarter of CKD cases are caused by hypertension or vascular ischaemic changes. Multiple types of glomerulonephritis together make up 10-15% of CKD cases, while genetic causes are present in about 5% of cases. Other causes include urinary outflow obstruction, infections or drug-induced disease.

1.2.5 CKD mortality & morbidity

Impaired kidney function leads to multiple medical complications. The symptomatic burden and risk for medical complications increases as kidney function declines. Population-based studies demonstrate that mortality and cardiovascular morbidity increases substantially from CKD stage 3 onwards.[54] Metabolic complications can be detected from early stages of CKD and include development of insulin resistance or accumulation of pro-inflammatory cytokines. [55, 56] As CKD progresses to moderate and severe levels, patients become increasingly likely to develop anaemia, mineral bone disease, electrolyte disturbances, acidosis and fluid retention. [46, 54] Uraemia describes the build-up of toxins in the blood that occurs when renal filtration becomes insufficient, leading to symptoms such as fatigue, reduced appetite, nausea, weight loss, muscle cramps, itching and changes in mental status.

1.2.6 CKD principles of current management

Treatment for CKD focuses on preserving residual kidney function and controlling symptoms. Addressing the underlying cause or disease, alongside optimising cardiovascular risk factors, are central pillars for reducing progression of CKD to more advanced stages.[57] Additionally, angiotensin blockade and SGLT2 inhibitors are efficacious drugs for reducing progression of CKD, especially in proteinuric kidney disease.[58] Management for CKD-related complications includes a range of medications alongside dietary modifications to correct or limit anaemia, mineral bone disease, acidosis, electrolyte disturbances and fluid overload.[47] At the point of end-stage renal failure, renal replacement therapy in the form of dialysis or kidney transplantation are the ultimate resort for sustaining life.

In summary, CKD is a very common disease with rising prevalence in the elderly population. Impairment in kidney function is accompanied by increased mortality, complex metabolic alterations and complications affecting nearly all organ systems of the body. Current management approaches aim to limit progressive loss of kidney function, which is irreversible. Dialysis or transplantation remains a last resort for people with end-stage kidney failure.

1.3 Sarcopenia in CKD – the importance of the problem

1.3.1 Prevalence of sarcopenia in CKD

People with CKD are at greater risk of sarcopenia than the general population. Prevalence estimates for sarcopenia specifically in CKD populations range from 4% to higher than 50% according to several reviews on this topic.[59-61] The wide range in estimates reflects heterogeneity in study populations (e.g. age, CKD severity or comorbidities) and applied diagnostic criteria for sarcopenia. A large cohort study among relatively healthy adults (UK biobank) demonstrated that people with CKD stage 3-4 are roughly twice as likely to have low grip strength than people without CKD.[26] A rise in sarcopenia prevalence with declining renal function has been reported by several studies.[26, 62] Accordingly, sarcopenia is particularly common among people with end-stage renal failure. A recent meta-analysis found a pooled prevalence of sarcopenia of 25.6% (95% C.I. 22.1-29.4%) among patients on dialysis. Therefore, a well-developed body of evidence highlights that CKD is strongly associated with sarcopenia and that sarcopenia affects a substantial proportion of patients with CKD.

Comparisons of sarcopenia prevalence between populations with and without CKD face certain methodological challenges. Crude comparisons of prevalence values reported in different studies are rarely valid given the large influence of applied diagnostic criteria, age, comorbidities and care setting on diagnosing sarcopenia. Even when studies directly assess sarcopenia prevalence according to kidney function, care must be taken to exclude selection bias between the groups. Another potential bias relates to the way in which renal function is commonly determined in clinical practise. Serum creatinine is lower in sarcopenic populations

and therefore glomerular filtration rate will be overestimated, which would reduce the likelihood of detecting associations between CKD and sarcopenia.[63] These potential biases pose a challenge when it comes to precise and reliable quantification of the excess risk for sarcopenia attributable to CKD. Furthermore, these limitations may explain inconsistent findings in some studies that do not show an association between CKD and sarcopenia. Nevertheless, the evidence presented above was carefully selected to have mitigated for these potential biases as far as possible, strengthening the validity of a significant association between CKD and sarcopenia.

1.3.2 Impact of sarcopenia in CKD

As in the general population, sarcopenia predicts increased mortality among patients with CKD. This has been confirmed in several meta-analyses of studies involving pre-dialysis and dialysis-dependent cohorts.[64-67] Patients with co-existing low muscle mass, strength or function on average have 1.5-2.4x higher adjusted mortality risk than patients without signs of sarcopenia. It appears that the relative mortality risk related to sarcopenia is similar in CKD and non-CKD populations.[68] Yet, the excess mortality risk related to declining kidney function itself leads to a compound effect between CKD and sarcopenia.[69] Hence, sarcopenia in CKD associates with higher absolute increments in mortality than in the general population.

A wide range of other adverse health outcomes have been associated with sarcopenia in patients with CKD. Patients at CKD stage 3-4 with low muscle strength had 2-fold higher risk (95% CI: 1.5-2.7) to develop end-stage renal failure.[26] A meta-analysis confirms that

cardiovascular events were significantly more common in dialysis patients with sarcopenia (adjusted OR 3.80 (95% CI 1.79 to 8.09).[67] Further complications associated with sarcopenia in CKD include osteoporosis, functional disability, hospitalisation and lower quality of life.[59, 70-72]

Summing up evidence from the literature, it has been robustly established that sarcopenia is common in CKD, that CKD itself is a risk factor for sarcopenia and that presence of sarcopenia predicts major adverse outcomes for people with CKD. Prevention and treatment of sarcopenia in CKD is therefore an important priority. This is echoed by patients with CKD and their relatives, who have ranked *fatigue* and *life participation* as two of their top five priorities for care in carefully conducted survey studies.[73] Furthermore, better prevention and treatment of systemic complications of CKD has been highlighted as a priority for research in the UK Renal Research Strategy, a consensus document distilling views from patients, health professionals and charitable organisations.[74] For these reasons, in combination with gaps in our knowledge on pathophysiology and lack of effective treatment options that will be discussed in the next section, there is a strong justification to invest in research on sarcopenia in CKD.

1.4 Pathophysiology of sarcopenia in CKD and existing mitigation strategies – the need for novel approaches to address anabolic resistance

1.4.1 Changes in skeletal muscle in CKD

Both muscle mass and muscle strength are decreased in chronic kidney disease.[26, 75-77] Decline in muscle strength or muscle function can be more prominent than reduction in muscle size, mirroring age-related muscle changes in the general population.[78, 79] Patients with end-stage renal failure have lower strength and slower gait speed even though total muscle cross-sectional area may be similar to age-matched healthy controls.[80] Importantly, muscle strength and function are stronger predictors for adverse clinical outcomes such as hospitalisation or mortality in CKD populations than muscle mass alone, as is the case for sarcopenia in general.[65, 72, 81, 82]

One possible explanation for greater loss of muscle strength than mass is impaired neuronal activation of contraction. Few studies have characterised electrophysiological changes of the neuromuscular unit in CKD with inconsistent results.[75, 83, 84] Equally, it remains unknown whether decreased supraspinal stimulation of movements contributes to muscle weakness in CKD. These remain areas for further research.

Morphological and histological changes that impair muscle force generation in CKD are better understood. The proportion of non-contractile tissue within skeletal muscle increases in CKD.[80, 85, 86] This occurs alongside atrophy of muscle fibres. Type II muscle fibre cross-sectional area is consistently smaller compared to healthy controls, with some reports

suggesting that type I fibres may also reduce in size.[83, 87, 88] Non-contractile tissue within skeletal muscle commonly represents infiltration with lipids.[89] Furthermore, functional muscle tissue is replaced with fibrotic tissue in CKD.[90, 91] Finally, microvascular rarefication limits the capacity for exchange of nutrients, metabolites and oxygen within skeletal muscle of patients with CKD.[88, 92, 93] These changes collectively reduce the quality of skeletal muscle in CKD and impede force generation.

Muscle fibre atrophy and ultimate reduction in muscle size are the consequence of an imbalance in protein synthesis and protein degradation. Myocellular protein stores are dynamically broken down and renewed in normal physiology. Experiments with labelled amino acids consistently showed depressed rates of net protein synthesis and elevated rates of net protein degradation in patients and animal models of CKD.[94-96] These changes drive net protein loss and muscle fibre atrophy in CKD.

Muscle protein degradation occurs via several mechanisms that act in concert. These mechanisms include the ubiquitin-proteosome system (UPS), caspase 3 and autophagy. The ubiquitin-proteosome system is an efficient and specific intracellular machinery of enzymes that label proteins with polyubiquitin chains for degradation. [97] Fbxo32 and Trim63 are important examples of E3 ubiquitin ligases in muscle. A multi-subunit protease complex then cleaves ubiquitin-labelled proteins into small peptides or amino acids. Caspase 3 possesses proteolytic activity to break up large protein structures such as actomyosin complexes, thereby facilitating disassembly of sarcomeres.[98] Autophagy describes a process whereby intracellular structures such as organelles or proteins in bulk are engulfed within an

autophagosome vesicle.[99] Upon fusion with a lysosome, the contents of the autophagosome are degraded by acidic proteases. Extensive evidence has shown that activity of the UPS, caspase 3 and autophagy are elevated in skeletal muscle in conditions of CKD, contributing to excessive protein degradation and muscle atrophy.[100]

Analogous to protein degradation, protein synthesis activity and muscle growth is a compound result of multiple mechanisms. These mechanisms include anabolic insulin/IGF-1 signalling, ribosomal function and regenerative satellite cell function. The hormones insulin and IGF-1 bind receptors in the cell membrane to activate the PI3K-Akt-mTOR signalling cascade.[101] This results not only in upregulation of cellular growth and protein translation, but also mediates concurrent downregulation of autophagy and the UPS system. Production of new proteins via protein translation occurs at the ribosome. Ribosomal efficacy for protein translation is modulated through phosphorylation of translation initiation factors (4E-BP1 or eIF4G) and translation elongation factors (S6K1 or ERK1/2 and their targets).[100, 102] Satellite cells represent muscle progenitor cells with critical roles for regeneration and maintenance of muscle mass.[103, 104] Growth factors such as IGF1 stimulate proliferation, differentiation and fusion of satellite cells with myofibers, thereby refreshing the myofiber pool.[103, 104] All of these central processes for protein synthesis and muscle growth, namely insulin/IGF-1 signalling, ribosomal efficacy and satellite cell function, appear to be suppressed in conditions of CKD.[100]

Taken together, there is an emerging understanding of altered activity in effector mechanisms that determine elevated rates of protein degradation and suppressed rates of protein synthesis

in skeletal muscle in CKD. However, the aetiological factors that link CKD and altered protein metabolism in muscle are much less clear, leaving significant gaps in our knowledge on the pathophysiology of sarcopenia in CKD.

1.4.2 Physical activity

Physical activity, producing mechanical stimulation of muscle, is important for maintaining and increasing muscle mass and strength. A large proportion of patients with CKD engage in less physical activity than recommended in general population guidelines.[105] Sedentary time is higher in people with CKD than people without CKD, and physical inactivity becomes more pronounced with advancing stages of CKD.[106-108] The reasons for physical inactivity are not fully clear, but may include metabolic complications, cardiopulmonary dysfunction, mental disease burden and treatment-related immobilisation. Several studies have highlighted low physical activity as a risk factor for sarcopenia in CKD.[26, 109]

Physical activity and exercise is recommended for patients with CKD according to consensus guidelines.[110] Several studies have suggested that exercise interventions can have a positive impact on measures of sarcopenia. A recent meta-analysis concluded that intra-dialytic exercise improved measures of muscle strength and function, but not muscle mass in patients on haemodialysis.[111] Nevertheless, the exercise trials in CKD to date frequently vary in terms of study cohorts, interventions and outcome measures, leading to inconsistencies in the available evidence.

There are important limitations with exercise as a sole management strategy for sarcopenia in CKD. Firstly, patients with CKD are commonly less tolerant of physical activity than people without CKD.[112] Secondly, physical exercise may be less effective in people with CKD to achieve benefits in terms of muscle strength or growth.[113-115] This deficit probably reflects underlying alterations in muscle metabolism, such as anabolic resistance and upregulated protein degradation, that are not addressed by exercise alone.[116, 117]

1.4.3 Nutrition

Meeting nutritional requirements can be a challenge for some patients with CKD. On the one hand, multiple risk factors for inadequate dietary protein and energy intake are present in CKD. These include altered taste and nausea due to uraemia, disturbances in appetite-regulating hormones (e.g. leptin, ghrelin), early satiety with delayed gastric emptying, and anorexia related to intercurrent illness or comorbidities.[118] On the other hand, CKD represents a hypermetabolic state with high utilisation and losses of nutrients. Metabolic complications such as acidosis and inflammation, acute illnesses and hospitalisation, and unavoidable losses of circulating nutrients during dialysis all contribute to higher nutritional requirements in CKD.[119] The factors promoting malnutrition become more prominent with advancing stages of CKD, meaning that people with low renal function or those on dialysis are at greatest risk of nutritional deficits.

Addressing nutritional deficits may help to prevent sarcopenia in CKD. Adequate nutrient supply is a pre-requisite for growing muscle size in response to anabolic stimuli.[4, 25] Unsurprisingly, markers of malnutrition predict sarcopenia risk in cohorts with CKD.[59]

Supporting patients with CKD who are at risk of malnutrition to achieve adequate nutritional intake is undoubtedly important. Yet, the evidence for nutritional interventions to correct sarcopenia is mixed, particularly if nutritional interventions are implemented in isolation. Trials with oral protein supplementation alone found no significant improvements in muscle mass or strength in patients on dialysis or older adults in general.[111, 120] Several studies have tested supplementation of specific nutritional components in CKD. There are reports that amino acid supplementation may lead to benefits in muscle mass or muscle strength.[111, 121, 122] In view of small sample sizes and heterogeneous results, these findings will require further validation.

Overall, nutritional interventions seem unlikely to offer a general remedy against sarcopenia in CKD. Their efficacy in clinical trials appears limited, especially when implemented in isolation. This may reflect a relative nutritional adequacy in many patients with CKD. Patient selection for nutritional interventions is likely critical, as those with greatest nutritional deficit are most likely to benefit. Furthermore, underlying metabolic derangements and anabolic resistance in CKD has many more facets than altered nutrition, leaving nutritional interventions alone insufficient to restore normal muscle physiology.[123]

1.4.4 Acidosis

Acidosis has been described as an important cause for muscle wasting in CKD.[124, 125] As renal function declines below a critical level, renal capacity for acid-base homeostasis is compromised and metabolic acidosis ensues. Experimental acidosis increases muscle protein degradation in otherwise normal rats, while correction of acidosis in rat models of CKD

reverses heightened muscle protein degradation.[125, 126] Protein degradation induced by acidosis has been linked with increased expression of ubiquitin and other components of the UPS.[127, 128] Furthermore, systemic acidosis may induce direct tissue damage and act as a pro-inflammatory stimulus.[129-131] Besides catabolic actions, acidosis also induces resistance to anabolic insulin signalling (see section 1.4.6 for more detail). The proteolytic and anti-anabolic effects of acidosis on skeletal muscle tissue have subsequently been validated in human studies.[124, 132] Overall, acidosis appears to play a significant role for muscle wasting in CKD.

The highest level of evidence for acidosis as a driver of muscle tissue loss comes from clinical trials. Several clinical trials have examined administration of bicarbonate or other acid-lowering therapies in populations with CKD. Correction of acidosis with oral bicarbonate improved measures of muscle mass in two randomised controlled trials.[132, 133] Furthermore, treatment with veverimer, a novel non-absorbed acid-chelating agent, corrected acidosis and improved physical function measures compared to placebo.[134-136] These improvements occurred without concurrent exercise or nutritional interventions, indicating direct effects of acidosis status on muscle metabolic balance. Indeed, clinical trials have demonstrated suppressed whole-body protein degradation and lower myostatin levels with correction of acidosis.[133, 137] Acidosis therefore is an important determinant of muscle metabolic changes in CKD, contributing to increased proteolysis and anabolic resistance.

1.4.5 Inflammation

Inflammation is elevated in CKD and adversely affects muscle metabolism.[138] Pro-inflammatory stimuli in CKD include metabolic acidosis, accumulation of circulating cytokines, oxidative stress, susceptibility to infections, microbiome changes and dialysis therapies.[100] Observational studies consistently describe rising levels of circulating inflammatory cytokines (e.g. CRP, IL1, IL6, TNFα) with declining kidney function.[55] These cytokines can act directly on muscle cells or via indirect pathways to promote muscle atrophy in multiple ways (reviewed in [139]). For example, TNFα activates the NF-κB signalling pathway, which promotes proteolysis via the E3-ubiquitin-ligases TRIM63 & FBXO32 and antagonises the anabolic insulin/AKT/mTOR pathway. Furthermore, IL6 activates JAK/STAT signalling, leading to upregulation of myostatin, an autocrine inhibitor of muscle growth. Hence, pro-inflammatory signals are capable to upregulate proteolysis mechanisms as well as impede anabolic signalling.

Several observational studies have linked inflammation with muscle loss and weakness in human CKD. A study among dialysis patients demonstrated a negative correlation between serum IL6 and visually graded muscle atrophy.[76] Another study in a pre-dialysis CKD population show a negative correlation between CRP and grip strength.[26] Such associations between inflammation and sarcopenia markers have been confirmed in systematic reviews.[59, 67] Finally, there is direct evidence from radiolabelled tracer studies that CRP level in haemodialysis patients associated with net forearm skeletal muscle protein balance, even after adjustment for clinical and demographic confounders.[138] Taken together, there is robust evidence for an association between inflammation and sarcopenia in human CKD.

A limitation with existing human studies on inflammation and sarcopenia is that they are largely associative by nature. There is little direct proof of a causal link in humans, even though a clear mechanistic basis has been established in preclinical studies. Likewise, there is an absence of evidence on potential beneficial effects of anti-inflammatory therapies to prevent or treat sarcopenia in CKD.

1.4.6 Insulin resistance

IGF1/insulin signalling is critical for regulation of muscle protein metabolism. IGF1 and insulin act via tyrosine kinase receptors and the secondary messengers IRS1, PI3K and AKT. Phosphorylated AKT stimulates anabolic processes via activation of mTOR complex 1 and downregulates catabolic processes, including FOXO1 and FOXO3 signalling, UPS activity and autophagic activity.[100]

Crucially, IGF1/insulin post-receptor signal transduction is impaired in CKD.[140, 141] Insulin resistance is evident in humans with CKD from an early stage of impaired kidney function and occurs principally in skeletal muscle tissue. There is direct evidence that insulin resistance in CKD has significant implications for muscle protein metabolism. Garibotto et al. showed that high and low dose insulin infusions decreased forearm net muscle protein balance and protein degradation in healthy people, only high but not low dose insulin infusion gave this effect in patients with CKD.[95]

The aetiology of insulin resistance in CKD is complex, with inflammation and acidosis being principal drivers.[56, 142] Inflammatory cytokines like IL6 induce expression of SOCS3 via the

afore mentioned JAK/STAT pathway. SOCS3 in turn reduces levels of the secondary messenger IRS1. Acidosis lowers the binding affinity of the insulin receptor for its ligand.[143] In addition, acidosis leads to inhibition of PI3K and mTOR activity, via a mechanism of reduced intracellular availability of branched-chain amino acids.[144, 145] Taken together, inflammation, acidosis and insulin resistance are interacting factors that adversely affect muscle protein homeostasis and promote atrophy in CKD. Despite recognising insulin resistance as an important contributor to sarcopenia, few studies have examined insulin-sensitising approaches to ameliorate sarcopenia in CKD without conclusive results.

1.4.7 Hormones

This section explores current knowledge about androgens, Vitamin D and growth hormone in CKD and the contribution of these hormones to excess sarcopenia risk. A separate in-depth discussion on glucocorticoid hormones will follow in subsequent sections, reflecting the prominence of this subject for this PhD.

1.4.7.1 Androgens

There is evidence for relative androgen deficiency in CKD.[146, 147] Men on haemodialysis commonly have low testosterone levels and features of hypogonadotrophic hypogonadism.[148] Testosterone has important anabolic effects on skeletal muscle, where it promotes protein synthesis, suppresses protein degradation, stimulates muscle fibre hypertrophy and inhibits the pathways to intramuscular lipid deposition.[149, 150]

Accordingly, testosterone levels have been associated with sarcopenia risk in CKD populations.[26]

Several randomised clinical trials have tested the effects of anabolic steroids in patients on haemodialysis. Nandrolone decanoate increased lean body mass and muscle function over 6 months.[151] Oxymetholone, another anabolic steroid, improved fat-free mass, grip strength and physical function over 24 weeks.[152] Data on anabolic steroid effects in the pre-dialysis CKD population are not available. Despite the promising results, the adverse effect profile of anabolic steroids has unfortunately limited their utility in clinical practise and precluded their wider use. Treatment-related complications include virilisation and hirsutism in women, testicular atrophy and infertility in men, as well as adverse cardiovascular changes.[153]

Separation of desirable androgen effects (muscle anabolism) and undesirable adverse effects (prostatic hypertrophy, virilisation, increased cardiovascular risk) has been attempted through the use of selective androgen receptor modulators (SARMs). These agents have relatively higher agonist activity in skeletal muscle tissue than in reproductive tissues, exploiting the differential expression of androgen receptor regulatory proteins.[45] Results from early Phase I/II clinical trials in non-CKD populations have been encouraging, showing increases in lean body mass.[154, 155] However, subsequent clinical studies failed to confirm meaningful improvements in muscle strength or function, while concerns regarding altered lipid profiles and hepatotoxicity persisted.[156, 157] This has stalled the licencing of SARMs as treatments for sarcopenia. Clinical data on SARMs in patients with CKD is not available.[150]

1.4.7.2 Vitamin D

Disturbances in vitamin D metabolism are commonplace in CKD, causing a deficiency in activated 1,25-vitamin D.[46] Vitamin D is an important physiological regulator of muscle function.[158] It controls myokine secretion, myogenic differentiation and muscle fibre hypertrophy. Observational studies have indicated a correlation between low vitamin D levels and risk of sarcopenia.[159, 160] Controversy remains however to what extent vitamin D supplementation may improve sarcopenia in CKD. Although one trial has shown improvements in physical performance in CKD patients with low vitamin D levels at baseline [161], other trials have failed to achieve improvements in muscle-related outcome measures.[162, 163] Given that vitamin D supplementation is already highly prevalent in CKD populations to limit mineral bone disease, it is dubious whether revision of current clinical practice for vitamin D supplementation could deliver further incremental benefits for sarcopenia prevention in CKD.

1.4.7.3 Growth hormone

Another hormone that has received attention in relation to sarcopenia in CKD is growth hormone. Growth hormone and its indirect effects via IGF-1 are important regulators of muscle size and strength, stimulating muscle growth and hypertrophy.[164] Uraemia, pro-inflammatory cytokines, acidosis and other factors precipitate relative resistance to the anabolic actions of growth hormone.[165, 166] Early clinical trials with recombinant human growth hormone in chronic haemodialysis patients gave promising results.[167-169] The treatment was well tolerated with an increase in lean body mass and reduction in adverse inflammatory and cardiovascular markers. A subsequent large Phase III study was

unfortunately terminated early due to logistical challenges and slow recruitment, without confirming improvements in lean body mass, physical performance or quality of life.[170] The value of recombinant growth hormone as a therapy for sarcopenia in CKD therefore remains uncertain, pending further clinical evaluation.

1.4.8 Myostatin, activin A

Myostatin is an important negative regulator of muscle growth. A member of the TFG-beta superfamily, myostatin binds to the transmembrane ActRIIB receptor in an autocrine fashion.[44] Its effects include suppression of protein synthesis, e.g. through dephosphorylation of Akt, and inhibition of satellite cell function. Studies in humans have linked rising levels of circulating myostatin with declining eGFR.[171] Furthermore, myostatin expression in skeletal muscle biopsies from patients with CKD stage 5 was associated with loss of muscle mass.[172] Myostatin blocking strategies have been effective against muscle atrophy and muscle fibrosis in animal models of uraemia.[173, 174] However, results in Phase II clinical trials to date have failed to fulfil the high expectations set in this therapeutic approach. Although some studies show moderate improvements in muscle mass, these changes were not accompanied by improvements in muscle strength or function.[44] Only one clinical study has examined myostatin inhibition in patients with CKD, but development of candidate pharmaceutical has been halted and full results have not been published (NCT01958970).

A recent report implicated activin A, another member of the TNF-beta superfamily that shares receptor activity with myostatin, in muscle atrophy in kidney disease.[175] Activin A was found to be overexpressed in kidney biopsies from patients with CKD. In mouse models of renal

impairment, muscle specific inhibition of activin A signalling was protective against muscle atrophy. This illustrates an intriguing crosstalk between kidney fibrosis and muscle atrophy. The approach of targeting activin A/ myostatin signalling to prevent sarcopenia in CKD awaits further evaluation in translational and clinical studies.

1.4.9 microRNAs

MicroRNAs are emerging as potential contributing factors to sarcopenia in CKD. Certain microRNAs have been identified as regulators of muscle metabolism and homeostasis.[100, 176] Examples include miRNA-26a, inhibiting FoxO and PTEN, miRNA-27a, suppressing myostatin, and miRNA-23a, lowering Fbxo32 and Trim63. Studies have suggested that these miRNAs are low in conditions of renal impairment.[100, 176] Further research will need to clarify to what extent these changes are significant for sarcopenia in human CKD and whether they can be targeted for therapeutic benefit.

1.4.10 Gaps in current knowledge and need for further research

The pathophysiology of sarcopenia in CKD is complex and incompletely understood. It is clear that CKD promotes loss of muscle mass, strength and function, accompanied by changes in skeletal muscle architecture and metabolic regulation. A fundamental shift in protein balance favouring accelerated rate of protein degradation and impaired response to anabolic stimuli has been clearly established. Physical inactivity, nutritional deficits, metabolic acidosis, inflammation, insulin resistance, hormonal changes and more have been implicated in the pathophysiology of sarcopenia in CKD, but their relative significance and interactions remain

poorly understood. Furthermore, several important mechanistic insights rely primarily on data from preclinical studies, missing further validation in humans so far. Further research therefore will benefit from integrating investigation of multiple aetiological factors to assess their relative importance and interactions, ideally also translating insights about pathophysiology to humans.

Identification, development and implementation of effective therapies against sarcopenia in CKD has been challenging. Treatment options at present are limited, often hampered by poor patient adherence (physical activity), underwhelming efficacy (nutritional interventions, vitamin D) or adverse effect profiles (androgens). Evidence is strongest to recommend resistance-based physical activity, targeted nutritional interventions and correction of acidosis. With the exception of acidosis correction however, no therapeutic options are available at present to address the fundamental metabolic disturbances of elevated proteolysis and anabolic resistance in skeletal in CKD. Identification and testing of novel therapeutic approaches for this very indication therefore is an important priority.

1.5 Background: glucocorticoid hormones

The central subject for this PhD is evaluation of glucocorticoid hormone metabolism as a risk factor and potential therapeutic target for sarcopenia in CKD. Before laying out the current evidence on glucocorticoid signalling as a contributing factor to sarcopenia (section 1.6) and the impact of renal dysfunction on glucocorticoid physiology (section 1.7), the following section will first offer a general introduction to glucocorticoid actions, regulation, metabolism and association with mortality.

1.5.1 Overview of glucocorticoid actions

Glucocorticoids represent a family of endogenous pleotropic steroid hormones that are essential to life and regulate a wide array of critical physiological processes. The major active endogenous glucocorticoid in humans, cortisol, plays a crucial role mediating the body's response to a diverse array of stressors.[177, 178] Cortisol acts through binding to the glucocorticoid receptor, which is ubiquitously expressed and mediates a wide range of cell-specific effects.[179] These in turn influence blood pressure and circulation, glucose homeostasis, immune function, metabolism, tissue remodelling and cognition.[177] The homeostasis of cortisol is itself dynamic, involving multiple mechanisms. These include central regulation of adrenal gland synthesis and secretion via the hypothalamic- pituitary-adrenal axis (HPA), systemic and peripheral tissue metabolism (both activating and inactivating), terminal metabolic inactivation in the liver and excretion of metabolites in urine.[180-182]

Diversity of glucocorticoid actions in cells and tissues is achieved through modulation of signal transduction. The glucocorticoid receptor is found in the cytosol in the absence of ligand. Upon ligand binding, the receptor moves to the nucleus and forms complexes for signal transduction, either as a homodimer or with other proteins. Signal transduction occurs via three principal mechanisms.[164] Firstly, ligand-bound homodimers of the glucocorticoid receptor bind directly to DNA sequences with glucocorticoid-responsive elements. The ligand-receptor complex thereby acts itself as a transcription factor to promote or suppress RNA transcription (direct genomic action). Second, the ligand-bound receptor interacts with other transcription factors, e.g. nuclear factor-κB, and modulates their capacity to control RNA transcription (indirect genomic action). Third, ligand-bound receptor interacts with membrane-associated receptors or secondary messengers, e.g. PI3K, to alter signal transduction in other pathways (nongenomic actions). These diverse pathways for signal transductions facilitate a wide range of glucocorticoid effects and allow for context-specific signal modulation.[179, 183]

1.5.2 Regulation of circulating glucocorticoids by the hypothalamic-pituitary-adrenal axis

Levels of cortisol in circulation vary significantly throughout the day. Cortisol levels exhibit a circadian rhythm under regulation of the hypothalamic circadian clock.[178, 180] Levels are typically highest in the morning, declining throughout the day and reaching a nadir around midnight (Figure 1.2). On top of this circadian rhythm, cortisol levels exhibit ultradian fluctuations. This means cortisol levels rise and fall on time scales significantly shorter than 24 hours. Reasons for ultradian fluctuations include pulsatile nature of cortisol release from the adrenal glands, increments in cortisol following meals or with stress and other factors.

Consequently, levels of cortisol in blood for the same individual can vary significantly over 30 minutes.

Systemic levels of cortisol are under the control of a homeostatic mechanism involving the hypothalamus, anterior pituitary gland and adrenal glands [178, 180] (Figure 1.2). Corticotropin-releasing hormone (CRH) is secreted by the hypothalamus in response to circadian rhythm, stress and other stimuli. CRH reaches the anterior pituitary gland via the portal system, where it stimulates release of adrenocorticotropin hormone (ACTH). ACTH is transported in the bloodstream and affects adrenal gland function, where it stimulates production of cortisol and its release into the circulation. Besides regulation of adrenal steroidogenesis, ACTH also acts through nonsteroidal pathways by binding and activation of melanocortin receptors to influence metabolic function and immunomodulation.[184, 185] In the circulation, approximately 90% of cortisol is bound and sequestered to the corticosteroid-binding protein, and to a lesser extent (5%) albumin, with only around 5% of total cortisol existing as free ligand available for signalling.[186] Given its lipophilic properties, cortisol passes relatively unimpeded through cellular membranes where it elicits signal transduction through its binding to glucocorticoid receptor isoforms. Cortisol (or other synthetic glucocorticoids) mediates negative feedback at the hypothalamus and pituitary gland to suppress further CRH/ACTH release. The homeostatic set point for this negative feedback loop is subject to modulation by circadian rhythm, severe stress and factors such as circulating inflammatory mediators.

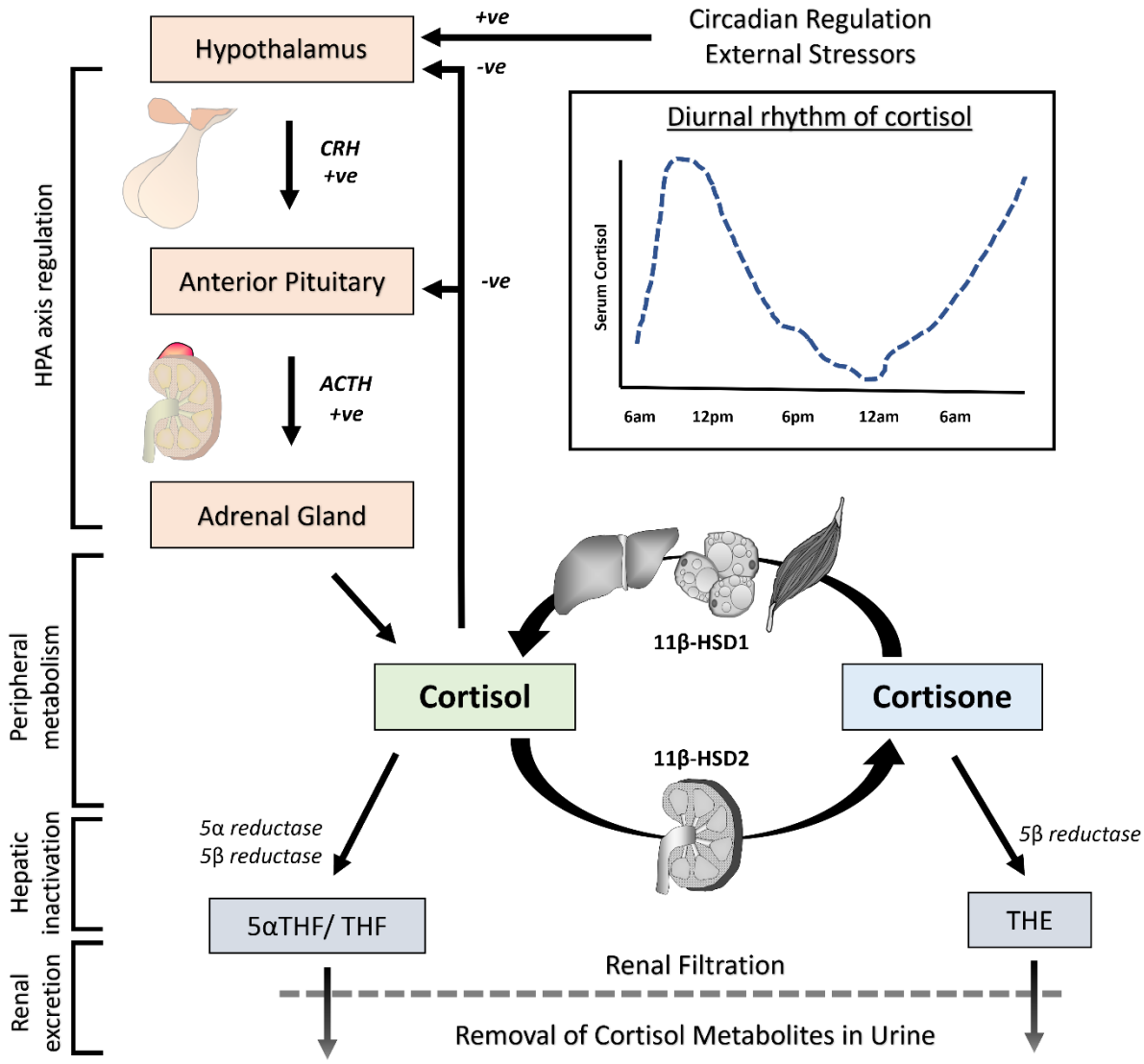


Figure 1.2 Pathways for physiological regulation of cortisol in the body.
The hypothalamic-pituitary-adrenal (HPA) axis controls cortisol synthesis in the adrenal gland and its release into the circulation. The activity of the HPA axis is set by the circadian clock, negative feedback from circulating cortisol, external stressors and other factors. Cortisol and inactive cortisone are interconverted by 11β-hydroxysteroid dehydrogenase (11βHSD) enzymes for reversible activation and inactivation in peripheral tissues. Liver, fat and skeletal muscle are predominant sites of cortisol activation by 11βHSD1, while kidney is the predominant site for reversible cortisol inactivation by 11βHSD2. Terminal inactivation of cortisol occurs in the liver through enzymatic reduction to tetrahydrocortisol (THF), 5α-tetrahydrocortisol (5α-THF) and tetrahydrocortisone (THE). These metabolites are ultimately removed from the body through filtration in the kidneys and excretion in urine.

The normal half-life of cortisol in the circulation is between 1-2 hours and is determined by several factors.[187, 188] These include its direct removal from the body through terminal metabolic inactivation in the liver and renal excretion in urine.[181] In this context, enzymatic clearance of cortisol in the liver involves reduction by 3α hydroxysteroid dehydrogenase and 5α reductase or 5β reductase to yield 5α-tetrahydrocortisol (5αTHF) or tetrahydrocortisol (THF) (cortisone is similarly converted to tetrahydrocortisone (THE)) (Figure 1.2). A fraction of tetrahydro-metabolites can also be further metabolized by the 20 α and 20 β reductases to α and β-cortols or cortolones. Lastly, conjugation reactions of metabolites with glucuronide increase the water solubility of glucocorticoid metabolites and facilitate urinary excretion.

1.5.3 Peripheral metabolism of glucocorticoids by 11β-hydroxysteroid dehydrogenase enzymes

Further factors that influence circulating cortisol half-life include their reversable metabolism by the enzymes 11β-hydroxysteroid dehydrogenase (11βHSD) type 1 and 2 [189] (Figure 1.2). These enzymes mediate the interconversion of active cortisol and inactive cortisone within peripheral tissues. These enzymes also allow for tissue-specific pre-receptor modulation of glucocorticoid signalling.

11βHSD type 1 predominantly activates cortisone to cortisol in the presence of its cofactor NADPH (nicotinamide adenine dinucleotide phosphate). High concentrations of NADPH are supplied by the enzyme hexose-6-phosphate dehydrogenase (H6PD) which co-localises with 11βHSD1 at the endoplasmic reticulum.[182] Intact cellular architecture is therefore important for 11βHSD1 to function effectively as an oxoreductase (i.e. cortisone to cortisol conversion),

whereas dehydrogenase activity (i.e. cortisol to cortisone conversion) predominates in tissue homogenates.

Expression of 11βHSD1 is high in metabolically active tissues such as liver, skeletal muscle and fat, with evidence for glucocorticoid-activating enzymatic activity in human studies.[189, 190] Furthermore, 11βHSD1 is expressed at lower levels in vascular tissue, bone, brain, myeloid and stromal tissues (its biological role in these systems will not be discussed in further detail; reviews with a detailed account on this topic are available [182, 189]). 11βHSD1 expression and activity is dynamically regulated in these tissues. Inflammatory cytokines TNFα and IL1 potently upregulate 11βHSD1 activity via the NF-kB signalling pathway.[191] 11βHSD1 activity is also induced by glucocorticoid stimulation itself, likely via the C/EBPβ pathway.[192] Finally, there is evidence to suggest that 11βHSD1 expression increases with aging and in metabolic diseases.[193, 194] Locally within tissues, 11βHSD1 allows autocrine and paracrine modulation of glucocorticoid signalling, principally through glucocorticoid activation and amplification of local glucocorticoid signalling. On a systemic level, 11βHSD1 also contributes to cortisol production from peripheral tissues, influencing rate of cortisol turnover.

In contrast, 11βHSD2 inactivates cortisol to cortisone in the presence of its cofactor NAD^+ (nicotinamide adenine dinucleotide) and is highly expressed in the kidneys. 11βHSD2 is considered important for preventing indiscriminate activation of the mineralocorticoid receptor (MR) by cortisol, which possesses a high affinity and agonist activity at the MR.[195] At the systemic level, there is considerable continuous interconversion between cortisol and cortisone that determines a circulating equilibrium for these steroids.[190, 196] Under resting

conditions, a roughly 10-fold lower level of total inactive cortisone is counterbalanced by a significantly lower affinity of cortisone for circulating binding proteins, resulting in comparable levels in serum free levels as is observed with cortisol.[181] Consequently, changes in 11βHSD1 or 11βHSD2 activity can modulate not only local tissue specific cortisol availability, but also influence systemic rates of cortisol appearance and disappearance.[197-199]

1.5.4 Cortisol and mortality risk

Both glucocorticoid deficiency (adrenal insufficiency) and glucocorticoid excess (Cushing's syndrome) are themselves causes of significant morbidity and mortality.[200, 201] Even a mild degree of persistent elevated cortisol exposure has a profound impact on mortality. Overall mortality and in particular cardiovascular mortality remains elevated in patients with treated Cushing's disease 10 years after remission compared to the general population.[202] Slight increments in hydrocortisone replacement dose for adrenal insufficiency translate into significantly increased mortality related to cardiovascular, respiratory and malignant disease.[203] And in patients with adrenal incidentalomas, evidence of elevated adrenal cortisol output in the absence of clinical features of Cushing's syndrome predicts mortality related to cardiovascular disease and infection.[204] Of interest, glucocorticoid excess possesses considerable overlap with the pattern of complications observed in CKD, e.g. hypertension, elevated cardiovascular risk, insulin resistance, dyslipidaemia, sarcopenia, osteoporosis, immune dysfunction or mood disturbances (Figure 1.3). This warrants consideration whether these similarities are co-incidental or share underlying biological links.

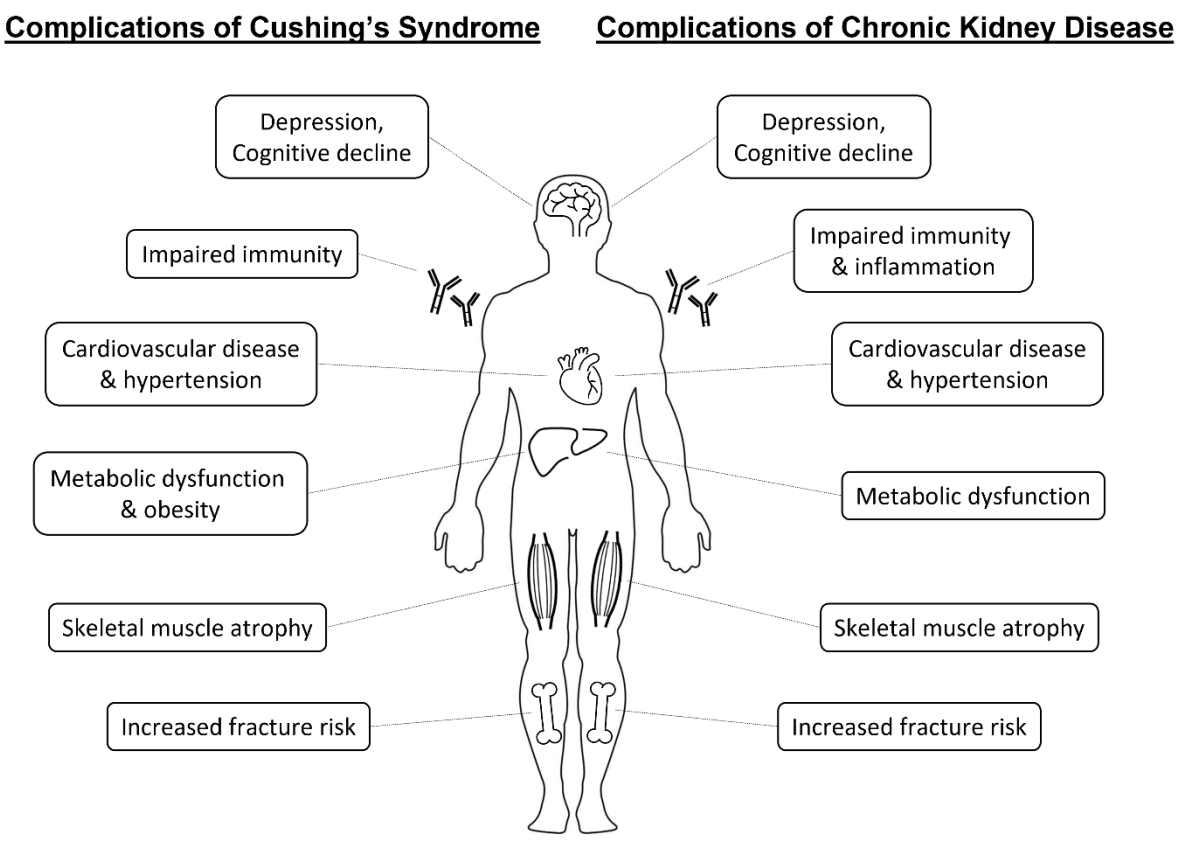


Figure 1.3 Similarities between systemic complications of Cushing's syndrome and chronic kidney disease

Specifically in adults with CKD, associations between mortality and cortisol have been investigated in a small number of studies (Table 1.2). Morning cortisol was associated with adjusted all-cause mortality in two cohort studies of patients on haemodialysis.[205, 206] The largest cohort study to date, involving 1255 patients with diabetes on haemodialysis, reported increased crude hazard ratios for all-cause and cardiovascular mortality with higher morning cortisol. However, these associations were not statistically significant in multivariable analysis.[207] Finally, a post-hoc survival analysis is described by Hou et al. for a music therapy intervention in haemodialysis patients. Interestingly, there was a trend for reduced all-cause mortality and significantly lower cardiovascular mortality in the subgroup of patients with

greater reduction in salivary cortisol following 1 week of music therapy compared to patients with little or no reduction in salivary cortisol.[208] Taken together, the available observational evidence is generally supportive of an association between increased cortisol levels and increased mortality among patients on haemodialysis.

Certain limitations must be acknowledged regarding the available literature on links between cortisol and mortality. Firstly, available reports are restricted to cohorts with end-stage renal failure. No data for patients with pre-dialysis CKD were available. Secondly, the available data is based on studies with observational design. Even though most studies conducted multivariable analyses to control for possible co-variables, confounding remains a risk in these studies. Thirdly, most studies relied on measurements of morning serum cortisol for investigating associations with mortality. In contrast, epidemiological studies have demonstrated that salivary cortisol measures or measures of diurnal variability in cortisol exhibit stronger correlations with all-cause mortality in the general population than morning blood cortisol levels.[209-214] Advancing the existing literature with studies using more sensitive markers of endogenous glucocorticoid signalling in relation to mortality remains an objective for future research.

Table 1.2 Associations between Cortisol and Survival in CKD

Citation	Study Type	Study Population	n total	Age	Male	Follow-up	At risk group	Control group	All-cause mortality	Cardiovascular mortality
Kim et al. (2022)	observational - cohort study	Haemodialysis patients	133	62.9 ± 10.5 (mean, s.d.)	49%	3.3 ± 2.0 years (mean, s.d.)	above median for pre-dialysis serum cortisol (>10µg/dL)	below median pre-dialysis serum cortisol (<10µg/dL)	adj. HR 1.234 (95% C.I. 1.022-1.49, p=0.029)	not reported
Hou et al. (2017)	interventional - post-hoc analysis in RCT	Haemodialysis patients	49	70.5 ± 8.0 (mean, s.d.)	49%	5 years	salivary cortisol reduction <0.6pg/ml with music therapy for 1 week	salivary cortisol reduction >0.6pg/ml with music therapy for 1 week	no significant difference (p=0.051)	lower survival (63.6% vs 81.6%, p=0.028)
Gracia-Iguacel et al. (2014)	observational - cohort study	Haemodialysis patients	75	64 ± 13 (mean, s.d.)	45%	1.7 years [0.7-2.6] (median, IQR)	high tertile of morning serum cortisol (≥18µg/dL)	low and middle tertile of morning serum cortisol (<18µg/dL)	adj. HR 1.16 (95% C.I. 1.027-1.309, p=0.017)	not reported
Drechsler et al. (2013)	observational - cohort study	Haemodialysis patients with diabetes	1255	66 ± 8 (mean, s.d.)	54%	4 years (median)	high quartile of serum cortisol (>21.1µg/dL)	low quartile of serum cortisol (<13.2µg/dL)	adj. HR 1.10 (95% CI 0.86-1.40)	adj. HR for CV mortality 1.31 (95% CI 0.99-1.73)

Abbreviations: RCT, randomised controlled trial; s.d., standard deviation; IQR, interquartile range; adj. HR, adjusted hazard ratio; C.I., confidence interval; CV, cardiovascular

1.6 Glucocorticoid effects on skeletal muscle – a risk factor for sarcopenia

1.6.1 Adverse effects of glucocorticoid signalling on skeletal muscle tissue

Glucocorticoids induce skeletal muscle atrophy, especially in the setting of chronic glucocorticoid excess. The earliest observations of glucocorticoids as a cause of muscle atrophy and weakness date back to the early 20th century and the description of patients with Cushing's syndrome, a condition of glucocorticoid excess. Myopathy is a clinical feature in more than half of these patients at presentation.[215] With the availability of exogenously administered glucocorticoids and ACTH in the mid-20th century, metabolic tracer studies provided direct evidence that glucocorticoids stimulate protein degradation and impair protein synthesis in skeletal muscle.[216] Another substantial leap in grasping the significance of glucocorticoids for muscle atrophy occurred in the 1980's onwards, when animal studies highlighted that muscle atrophy in response to a range of stimuli such as acidosis, fasting or sepsis depended on endogenous glucocorticoid signalling.[126, 217-219] Over the past decades, glucocorticoid-based in vitro and in vivo models of muscle atrophy have made a significant contribution to our understanding of canonical muscle atrophy mechanisms.[220-222] Exogenous glucocorticoid use has become widely recognised as an independent risk factor for muscle atrophy and weakness.[30, 223] Lately, even subclinical mild overactivity of endogenous glucocorticoid signalling is getting established as a cause of metabolic disturbance in skeletal muscle and risk factor for sarcopenia.[224-226]

Glucocorticoid-induced myopathy exhibits a characteristic pattern of changes in skeletal muscle. The most prominent feature on skeletal muscle biopsies is muscle fibre atrophy.

Atrophy is typically more pronounced for fast-twitching, glycolytic type II muscle fibres.[227-229] These changes reflect imbalances in muscle protein turnover, with glucocorticoid signalling inducing concurrent increase in protein degradation and decrease in protein synthesis.[216, 230, 231] Muscle structure otherwise appears intact, without evidence of fibre damage or cellular infiltrates. Along with muscle atrophy, people with glucocorticoid myopathy have impaired muscle strength and function, e.g. lower handgrip strength or prolonged chair rising test time.[232] Proximal musculature of the lower limbs is commonly affected.[233] Some reports suggest intramuscular fat infiltration, microvascular changes or electrophysiological disturbances may occur in glucocorticoid myopathy, although more detailed descriptions of these alterations are pending.[234-236]

Glucocorticoids act directly on skeletal muscle tissue to influence muscle phenotype and function. This has been established through extensive in vitro and in vivo research, including animal models with muscle-specific genetic alterations in the glucocorticoid receptor gene.[217, 237, 238] All three signal transduction pathways (direct genomic action, indirect genomic action and nongenomic action) are operational in skeletal muscle cells and contribute to glucocorticoid-induced myopathy.[222, 239]

Glucocorticoids exert potent anti-anabolic actions in skeletal muscle. Multiple mechanisms have been described in this regard. Importantly, glucocorticoids antagonise signal transduction of the IGF-1/insulin – IRS-1 – PI3K – Akt axis. This occurs at various steps, including accelerated degradation of IRS-1, upregulation of PI3K-inhibitor p85α and direct protein-protein interaction of PI3K with the glucocorticoid receptor.[217, 240, 241] Additionally,

glucocorticoids antagonise mTOR signalling. This involves upregulation of REDD1 and KLF-15, repressors of mTOR signalling, as well as reduction of intracellular branched-chain amino acids, which enhance mTOR activity. [242, 243] Finally, glucocorticoids also decrease production of IGF-1 by muscle, thereby further impeding protein synthesis and myogenesis.[244]

Glucocorticoids also exert profound catabolic actions in skeletal muscle. Upregulation of the ubiquitin-protein system in response to glucocorticoid stimulation has been characterised in numerous studies. Glucocorticoids increase expression of FOXO transcription factors, most notably FOXO-1 and FOXO-3a.[245, 246] FOXO transcription factors orchestrate a programme of gene expression that mediates muscle catabolism, influencing genes such as Trim63, Fbxo32, cathepsin-L, PDK4, p21 and 4E-BP1.[222] Besides indirect transcriptional activation of the E3-ubiquitin ligases Trim63 and Fbxo32 via FOXO, the glucocorticoid receptor itself additionally enhances Trim63 expression through direct transcriptional activation.[247] Catabolic actions of glucocorticoids that act through mechanisms other than the UPS have been studied to a lesser extent. Glucocorticoids have the capacity to increase myostatin production in muscle, with consequent inhibition of myogenesis and muscle atrophy.[248, 249] Accordingly, myostatin knock-out mice exhibit relative protection against glucocorticoid-induced myopathy.[250] In vitro experiments also suggest that glucocorticoids signalling stimulates autophagy, although further research is awaited to clarify the importance of these observations for overall glucocorticoid-mediated muscle changes.[251]

1.6.2 Glucocorticoids and muscle atrophy in CKD

It is readily apparent that there is considerable overlap between the skeletal muscle changes observed in CKD and the myopathic effects induced by glucocorticoid signalling. Yet, there is a striking scarcity of studies that have examined links between glucocorticoid signalling and sarcopenia in human adults with CKD. Garibotto et al. reported a correlation between muscle net proteolysis and plasma cortisol in 9 pre-dialysis CKD patients.[252] Metabolic acidosis correlated with both plasma cortisol and muscle net proteolysis in univariable analysis. Interestingly, only plasma cortisol and not acidosis remained significantly associated with proteolysis in multivariable regression. Therefore, metabolic acidosis may mediate changes in skeletal muscle metabolism via the glucocorticoid pathway. In support of this hypothesis, observations from human experimental studies show that endogenous cortisol production is upregulated in response to acute metabolic acidosis.[253, 254] However arguing against this hypothesis, muscle net proteolysis was reduced following correction of acidosis without changes in circulating cortisol in a trial with 16 patients on haemodialysis.[255] Another study with cross-sectional design found no significant differences in lean tissue mass in relation to morning serum cortisol levels among haemodialysis patients.[206] Based on the limited clinical data available in the literature, it is not possible to arrive at a conclusive answer about the role of endogenous cortisol signalling for altered muscle metabolism or sarcopenia in CKD.

A substantial body of preclinical evidence suggests that endogenous glucocorticoid signalling is critically important for skeletal muscle metabolism in conditions of renal failure. Glucocorticoids have a central role in shaping the effects of inflammation, acidosis and insulin

resistance on muscle metabolism. This significance of glucocorticoid signalling has been highlighted in several seminal animal experiments.

Experimental chronic metabolic acidosis in rats stimulates muscle protein degradation through a glucocorticoid-dependent mechanism.[126] Acidosis-induced protein degradation is prevented by adrenalectomy, i.e. elimination of endogenous glucocorticoid production, while supplementation with exogenous glucocorticoid restores the acid-induced proteolytic effect. This may be partly explained by upregulation of systemic glucocorticoid production, as evidenced by higher urinary corticosterone excretion with acidosis. Additionally, co-dependent synergistic upregulation of protein degradation by acidosis and glucocorticoids has been demonstrated in *ex vivo* and *in vitro* experiments, suggesting that aspects of the mechanism for synergistic action may be intrinsic to skeletal muscle tissue itself.[256, 257] Such an intrinsic mechanism may involve synergistic induction of resistance to insulin/IGF1 signalling.[258] The presence of renal impairment further adds to the complexity of interactions between acidosis, glucocorticoids and protein metabolism. Notwithstanding that correction of acidosis in rats with chronic uraemia reduces muscle protein degradation, acidosis correction does not reduce elevated corticosterone or correct impaired insulin-stimulated muscle protein synthesis.[125] Summing up these observations, acidosis and glucocorticoids have synergistic and co-dependent actions at multiple levels that promote muscle atrophy.

Glucocorticoid signalling is also required for mediating the myopathic effects of impaired insulin signalling. The actions of glucocorticoid to antagonise insulin/IFG-1 signal transduction have already been outlined above (see section 1.6.1). In addition, intramuscular glucocorticoid

stimulation is an essential co-factor for muscle atrophy in rats with streptomycin-induced insulin deficiency.[217] Insulin deficiency by itself did not stimulate muscle wasting in the context of muscle-specific glucocorticoid blockade. Another study showed that insulin resistance in different rodent models of chronic uraemia could be reversed through suppression of abnormal glucocorticoid activation.[259] These elegant in vivo studies highlight the relevance of endogenous, muscle-specific glucocorticoid signalling for anabolic resistance and muscle atrophy in relation to insulin resistance.

The interactions between glucocorticoids, inflammation and muscle metabolism are complex. Glucocorticoid signalling can be both beneficial or detrimental for skeletal muscle in the setting of inflammation, depending on the specific clinical or experimental circumstances.[260] Broadly speaking, in settings of intramuscular inflammation and inflammatory muscle injury, the anti-inflammatory actions of glucocorticoids may be beneficial. Examples include exogenous glucocorticoid therapy for Duchenne's Muscular Dystrophy or autoimmune myositis.[261, 262] Blockade of peripheral glucocorticoid signal amplification has also proven detrimental for muscle mass in a rodent model of inflammatory polyarthritis arthritis.[263] In contrast, myopathic adverse effects of glucocorticoids may exacerbate muscle atrophy in situations of systemic inflammation, regardless of their anti-inflammatory actions. In line with this, exogenous glucocorticoid use is an independent risk factor for patients with rheumatoid arthritis.[30] In rodent studies, the glucocorticoid antagonist RU 38486 reduced sepsis-induced muscle protein breakdown and UPS upregulation [218], and exogenous glucocorticoid therapy exacerbated muscle wasting in a polyarthritis model despite potent suppression of systemic

inflammation.[264] Hence, the consequences of glucocorticoid stimulation for skeletal muscle metabolism and phenotype are difficult to predict in the setting of co-existing inflammation.

Lastly, glucocorticoids and androgens exhibit reciprocal interactions in their effects on skeletal muscle. On a systemic level, upregulation of pituitary ACTH secretion not only promotes adrenal cortisol production, but also stimulates release of adrenal androgens (DHEAS, androstenedione).[265] Contrarily, systemic glucocorticoid stimulation significantly reduces circulating testosterone levels and skeletal muscle androgen receptor expression.[244] When it comes to regulatory pathways of skeletal muscle anabolism and catabolism, the actions of glucocorticoids and androgens appear diametrically opposed. While glucocorticoids suppress IGF-1, induce atrogens and inhibit Akt phosphorylation, testosterone can reverse these changes.[222] Bearing in mind these interactions between glucocorticoids and androgens, it comes as no surprise that gender can influence glucocorticoid effects on skeletal muscle. Indeed, several studies have shown sex-divergent changes in skeletal muscle in response to glucocorticoid stimulation.[183, 266, 267] Given these important interactions, efforts to characterise androgen-mediated regulation of skeletal muscle in a specific setting such as CKD will benefit from taking into account concurrent glucocorticoid signalling status.

In summary, glucocorticoid signalling critically influences how aspects of CKD such as acidosis, inflammation, insulin resistance or relative androgen deficiency impact on metabolism in skeletal muscle. However, there are significant gaps in our knowledge in terms of understanding the complex mechanistic basis for these interactions, validating these phenomena in humans and exploring opportunities for therapeutic targeting. Given the need

to advance knowledge on pathophysiology of sarcopenia in CKD and develop novel treatments to address anabolic resistance, there is a strong rationale for further research on glucocorticoid signalling in this context.

1.7 Changes in glucocorticoid balance in CKD – loss of kidney function promotes cortisol excess

Prior to embarking on research into glucocorticoid-mediated skeletal muscle changes in CKD, it is valuable to provide a comprehensive review of normal glucocorticoid physiology and how it may be perturbed by chronic renal impairment. The following section offers a detailed summary of existing literature on this topic, focusing on studies that were done in human adults.[1]

1.7.1 Measures of systemic cortisol levels in CKD

1.7.1.1 Morning cortisol in blood

Early morning blood cortisol level is the most frequently reported measure of systemic glucocorticoid exposure in CKD, endeavouring to measure cortisol level at its diurnal peak. There is however considerable variability between studies in the literature (Table 1.3). No statistically significant change of morning blood cortisol levels in CKD is reported by the majority of studies published since 1995.[268-284] Still, a substantial number of studies describe significant elevation of morning blood cortisol levels with renal impairment [285-291], while a few isolated studies also report a significant reduction.[292, 293]

[1] This description of changes in glucocorticoid physiology and metabolism in CKD forms part of a published literature review: Sagmeister MS, Harper L, Hardy RS. Cortisol excess in chronic kidney disease - A review of changes and impact on mortality. Front Endocrinol (Lausanne). 2022;13:1075809. The published article contains additional information and a description of the literature search methodology.

Multivariable regression analysis to assess potential confounding influences for correlations between serum cortisol and kidney function was performed in three studies.[286, 287, 290] Renal dysfunction was identified as the strongest predictor of higher serum cortisol in a cohort of patients with heart failure after adjustment for age, sex, body mass index (BMI), atrial fibrillation and diuretic use.[290] Furthermore, serum cortisol was independently and negatively correlated with estimated glomerular filtration rate (eGFR) after adjustment for age, sex, antihypertensive medications, BMI, blood pressure, total cholesterol and uric acid in a cohort of patients with hypertension.[287] In contrast, an independent correlation between serum cortisol and measured GFR was not apparent in the study by Vitolo et al. [286] This study however had significant methodological limitations for detecting such a relationship, as subjects with creatinine values outside the normal range were excluded from the study and the multivariable model for measured GFR included highly correlated blood urea nitrogen, renal diameter and renal plasma perfusion as independent variables besides serum cortisol.

The mixed results for early morning cortisol in CKD partly reflect limitations of this methodological approach to assess endogenous glucocorticoid physiology. The challenges relate to the volatility of morning cortisol levels and associated high measurement variability. [180] Morning cortisol levels not only vary substantially from person-to-person, but also have a brief window at their peak that is technically demanding to accurately predict. Studies reporting no change in morning cortisol levels on the whole relied on smaller study cohorts (median sample size = 24, range 11 – 149), making them more susceptible to these caveats. Statistical power to detect significant changes in the context of highly variable measurements was low for many of these studies. In contrast, studies that reported significant elevations of

Table 1.3: Morning Blood Cortisol Levels in CKD

Citation	Study Population	n	Finding
Studies reporting no significant difference for morning cortisol			
Asao et al. (2016)	Patients with Type 2 Diabetes (eGFR >15)	77	n.s.
Karu et al. (2016)	CKD stage 4	27	n.s.
Ruscher et al. (2015)	Haemodialysis patients & Healthy controls	18	n.s.
Raff et al. (2013)	Patients on Dialysis and Healthy Controls	24	n.s.
Zoladz et al. (2012)	Patients on Dialysis and Healthy Controls	43	n.s.
Deshmukh et al. (2005)	Patients with CKD and Healthy Controls	11	n.s.
Oguz et al. (2003)	Male Patients with CKD	60	n.s.
N'Gankam et al. (2002)	Patients on Dialysis and Healthy Controls	97	n.s.
Homma et al. (2001)	Patients on Dialysis, Patients with Diabetes and Healthy Controls	149	n.s.
Gunduz et al. (2001)	Patients with Renal Amyloidosis and Healthy Controls	37	n.s.
Jenkins et al. (1999)	Patients on Dialysis and Healthy Controls	24	n.s.
Clodi et al. (1998)	Patients on Dialysis and Healthy Controls	21	n.s.
van Herle et al. (1998)	Patients on Haemodialysis and Healthy Controls	11	n.s.
Morineau et al. (1997)	Patients on Dialysis and Healthy Controls	60	n.s.
Letizia et al. (1996)	Patients on Dialysis and Healthy Controls	34	n.s.
Vigna et al. (1995)	Patients on Dialysis for >10 years & Healthy Controls	17	n.s.
Fouque et al. (1995)	Patients on Dialysis and Healthy Controls	18	n.s.
Studies reporting higher morning cortisol in CKD			
Rodriguez-Gutierrez et al. (2021)	Patients with CKD (stage 3 to 5D)	60	negative correlation of cortisol with eGFR (p=0.002); trend for higher cortisol with advancing CKD groups (p=0.05)

Citation	Study Population	n	Finding
Vitolo et al. (2016)	Patients with Obesity (BMI>40 & normal creatinine)	50	negative correlation of cortisol with mGFR (p=0.03)
Li et al. (2016)	Patients with Hypertension (eGFR >30)	178	negative correlation of cortisol with eGFR (p=0.012); higher cortisol in low eGFR group (p=0.001)
Olsen et al. (2015)	Patients with Adrenal Adenoma (eGFR >30)	164	higher cortisol with eGFR 30-60 vs eGFR >60 (p=0.02)
Afsar et al. (2014)	Renal Outpatients	174	negative correlation of cortisol with creatinine clearance (p=0.015)
Armaly et al. (2012)	Patients on Haemodialysis and Healthy Controls	97	higher cortisol in dialysis group (p<0.001)
Guder et al. (2006)	Patients with Heart Failure (eGFR >15)	294	positive correlation of cortisol with CKD stage (p<0.001)
Chan et al. (2004)	Patients undergoing Creatinine Clearance Testing	82	higher cortisol with CrCl <20 (p=0.002)
Studies reporting lower morning cortisol in CKD			
Arregger et al. (2014)	Patients on Dialysis with Hypotension	80	lower cortisol in select subgroups of HD patients vs control group (p<0.05)
Svensson et al. (2002)	Patients with Type 1 Diabetes	29	lower cortisol in reduced eGFR group (p<0.05)

Abbreviations: e/m GFR – estimated/measured glomerular filtration rate; CrCl - creatinine clearance; n.s. - not statistically significant; BMI - body mass index.

morning cortisol with renal impairment tended to have larger study cohorts (median sample size = 130, range 50 – 294). Therefore, adequate sample size was a significant factor for the capacity of these studies to identify elevated morning cortisol in CKD.

The challenges related to determination of basal cortisol levels are made worse by lack of stringency with sample collection and laboratory assays. Even though conditions for sample collection and processing are detailed in most studies, there is no uniform procedure for all studies and hence variation in sample collection contributes to variation in results. In a minority of studies, conditions for sample collection were insufficiently stringent [272] or not reported at all [289], which severely restricts the interpretation of their results. Immunoassays

were used in most studies as the laboratory technique for measuring cortisol, with only a few studies utilizing mass spectrometry-based techniques.[268, 270, 275] Immunoassays have inferior specificity for cortisol and show significant inter-assay variability, meaning that this type of cortisol assay is more susceptible to interference from related metabolites and offers limited generalizability of results across studies.[294]

Heterogeneity in study populations is another important reason for diverging reports on early morning cortisol in CKD. Studies describing low cortisol levels in CKD contained biases towards populations with increased risk for adrenal insufficiency. These biases included participant selection based on intra-dialytic hypotension or co-existing type 1 diabetes. Associations between high morning cortisol and low renal function were more commonly described in studies that recruited participants with specified co-existing conditions, as opposed to studies that recruited participants based on CKD alone. Co-existing conditions in these instances were severe obesity, hypertension, adrenal adenoma or heart failure.[286-288, 290] Methodological differences between the two sets of studies cannot be excluded, e.g. a tendency for smaller sample sizes in studies with CKD as the principal inclusion criteria. Still, it is important to consider that morning cortisol levels are increased by renal impairment synergistically with other chronic conditions. Even at mild to moderate renal impairment, negative correlations between indices of glomerular filtration rate and morning cortisol level were evident in several cohorts with comorbidities.[286-288, 290] In this context, kidney function was an independent predictor for higher morning cortisol in multivariable regression analysis in cohorts with heart failure or hypertension.[287, 290] These observations are among the strongest arguments that loss of renal function is associated with higher morning cortisol.

Possibly, a synergistic effect between renal impairment and other chronic health conditions with a propensity to augment cortisol makes elevations in morning cortisol more prominent.

1.7.1.2 Evening and diurnal cortisol in blood

Evening, or late-night cortisol measurements, taken when diurnal levels are approaching their nadir in healthy individuals, provide an additional perspective on circulating cortisol levels. It has been established that such measurements are better suited to detect elevated cortisol exposure in conditions of mild to moderate glucocorticoid excess.[295] Significantly elevated levels of evening cortisol have been reported for several CKD cohorts (Table 1.4).[271, 273, 296] These studies involved patients across a range of CKD settings, including patients on renal replacement therapy. The most accurate measurements of cumulative cortisol exposure can be achieved by serial cortisol measurements over the course of the day, rather than one-off measurement in the morning or evening. Cortisol has been assessed in this way in CKD by a few small studies.[270, 271, 273] The diurnal rhythm of cortisol is usually recognisable in patients with CKD, but cortisol exhibits an attenuated rate of decline throughout the day, persisting at higher levels at the nadir compared to healthy individuals. No significant association between renal function and evening cortisol levels was reported in the study by Vitolo et al.[286] However, this study excluded participants with moderate or severe renal impairment, meaning this observation is in line with other studies showing more pronounced cortisol changes from CKD stage 3 onwards.[288, 297] In summary, the cumulative cortisol exposure in people with CKD appears higher over a 24-hour period, which is most apparent after the decline of the morning peak (Figure 1.4).

Table 1.4: Evening Cortisol Levels in CKD

Citation	Study Population	n	Finding
Studies reporting higher evening cortisol in CKD			
Cardoso et al. (2016)	Renal & Endocrine Outpatients and Healthy Controls	120	high evening cortisol in CKD 3 or 4 vs CKD 1 or control group by either diurnal measurement ($p<0.05$)
Raff et al. (2013)	Patients on Dialysis and Healthy Controls	24	high evening cortisol in CKD 5 (HD) vs control group by either diurnal measurement ($p<0.05$)
Martins et al. (2012)	Patients with resistant hypertension & failed dexamethasone suppression test	112	CKD more prevalent in group with 23pm salivary F>3.6nM than group with F<3.6nM
Deshmukh et al. (2005)	Patients with CKD and Healthy Controls	11	high evening cortisol in CKD 5 vs control group ($p<0.05$)
Studies reporting no significant difference for evening cortisol			
Vitolo et al. (2016)	Patients with Obesity (BMI>40 & normal creatinine)	50	n.s. (Note: proteinuria or creatinine outside normal range were exclusion criteria)
Ruscher et al. (2015)	Haemodialysis patients & Healthy controls	18	trend for high evening cortisol, but not statistically significant

Abbreviations: BMI - body mass index; HD – haemodialysis; F – cortisol.

1.7.1.3 Cortisol in saliva

Saliva cortisol levels are considered to represent serum free biologically active cortisol, meaning measures of salivary cortisol are less affected by variations in binding protein levels than measures of total cortisol levels in blood.[294, 298] Early morning salivary cortisol in CKD has been investigated by several studies and generally no statistically significant differences were found between CKD groups and control groups.[271, 280, 297] Reduced morning salivary cortisol level was reported in one study, albeit in a cohort of dialysis patients with a selection bias for recurrent hypotension.[292] In contrast, evening salivary cortisol is elevated in CKD according to two studies (Table 1.4). Cardoso et al. demonstrated higher evening salivary cortisol in patients with CKD stage 3 or 4 compared to control groups [297], while Raff et al.

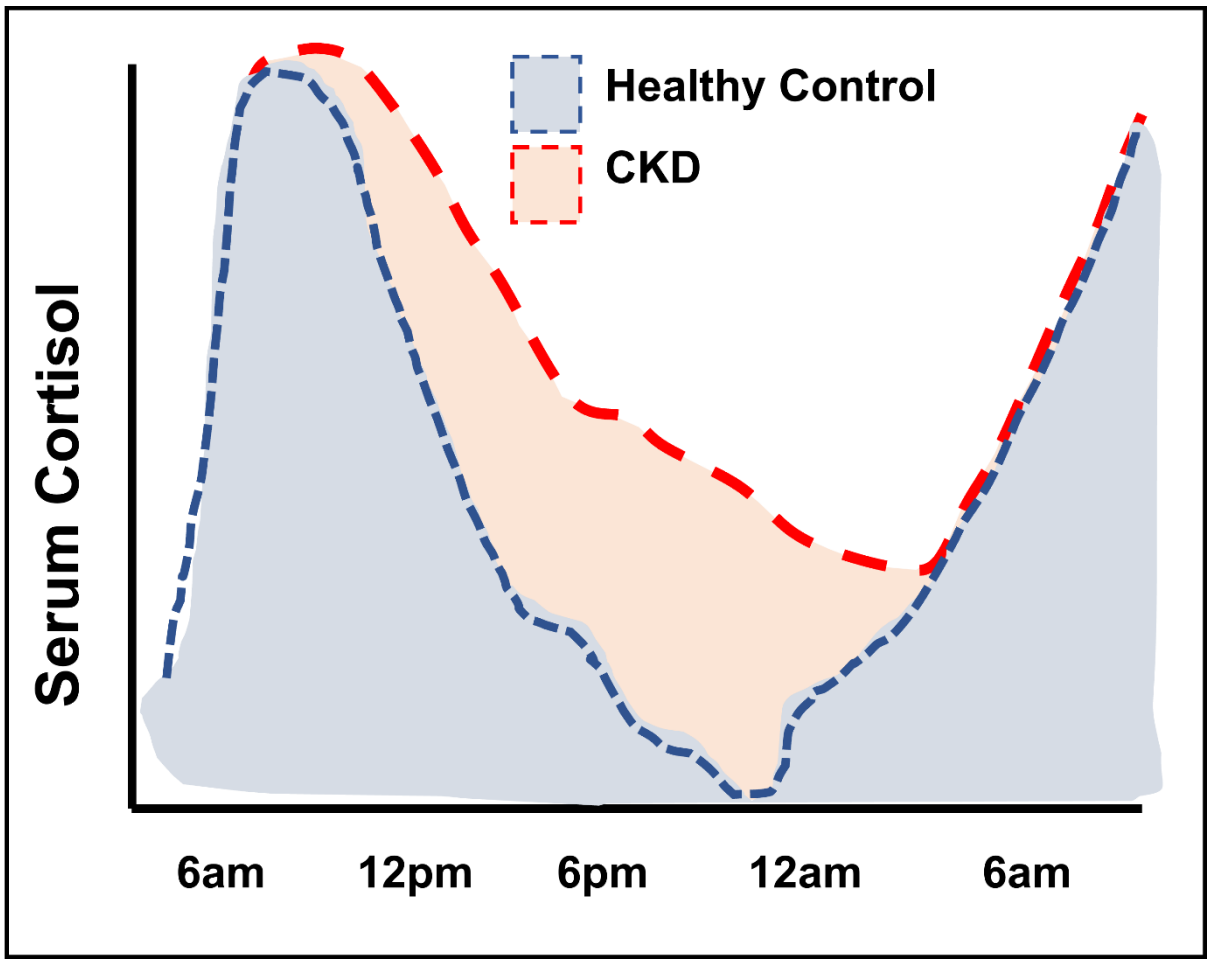


Figure 1.4 Illustration of diurnal cortisol levels in people with kidney failure compared to healthy control groups.
Figure adapted from Raff et al. [271] and Deshmukh et al. [273]

showed the same change for patients on haemodialysis.[271] The data from salivary cortisol measurements therefore shows parallels with data from blood cortisol measurements in CKD, with higher cortisol levels being prominent in evening-time measurements.

1.7.1.4 Cortisol and cortisol metabolites in urine

The total amount of cortisol secreted from the adrenal glands per day can be inferred from the sum of cortisol and its metabolites excreted in urine over 24 hours. Such data is available for patients with mild to moderate CKD. Is has been shown by several studies that the total

amount of glucocorticoid metabolites in 24-hour urine samples is similar to control groups [299-301] (Table 1.5). Gas chromatography-mass spectrometry was utilised in all three studies to determine cortisol metabolites in urine, representing a reliable and highly specific assay methodology. Details on urine processing, storage or addition of preservatives that may affect assay results were however not reported in the studies. Overall, the available data indicate that the total amount of cortisol secreted from the adrenal glands per day does not change in mild to moderate CKD.

Several putative explanations may reconcile this observation of unchanged total urinary cortisol metabolites with a pattern of increased endogenous cortisol exposure in CKD. Firstly, the control groups in all three studies examining urinary cortisol metabolites were not fully disease-free. The normal kidney function reference groups included people with treated hypertension [299, 300] or people with preserved kidney function in the context of proteinuria

Table 1.5: Cortisol and Cortisol Metabolite Measurements in Urine

Citation	Study Population	n	Finding
Studies reporting total glucocorticoid metabolites in 24-hour urine			
McQuarrie et al. (2013)	Patients with CKD or essential hypertension	100	n.s.
Henschkowski et al. (2008)	Patients with hypertension	163	n.s.
Quinkler et al. (2005)	Patients with CKD undergoing kidney biopsy	95	n.s.
Studies reporting unchanged cortisol excretion in urine			
Chan et al. (2004)	Patients undergoing creatinine clearance testing	82	lower urinary free cortisol with CrCl <60 than CrCl >60 ($p<0.001$); lower urinary free cortisol with CrCl <20 than CrCl 20-60 ($p<0.05$)
Oguz et al. (2003)	Male patients with CKD	60	lower urinary cortisol with advancing renal impairment ($p=0.00001$ for morning samples, $p=0.074$ for midnight sample)

Abbreviations: CrCl – creatinine clearance.

and/or haematuria.[301] Differences in urinary cortisol metabolite excretion in CKD may be less apparent for comparisons with these specific control groups instead of control groups that are genuinely disease-free. Secondly, prolongation of cortisol half-life in circulation can account for increased endogenous exposure in CKD in the absence of increased adrenal cortisol production (see section 1.7.3). In this context, unchanged urinary cortisol metabolite amount signifies inadequate negative feedback to downregulate adrenal cortisol production and maintain healthy systemic cortisol levels. In other words, the expected compensatory finding should be reduced urinary cortisol metabolites and unchanged urine cortisol metabolite amounts represent an abnormal response. Finally, the postulated drivers for HPA axis activation are likely less prominent in early CKD, namely inflammation, acidosis and other stress factors related to chronic disease. Changes in urine cortisol metabolites may accordingly be less readily detectable in the initial phases of CKD. Unfortunately urine cortisol metabolite quantification cannot be employed to gage adrenal cortisol secretion in end-stage kidney disease given the loss of urine output in this context.

Besides excretion of cortisol metabolites, a small fraction of unchanged cortisol is also excreted in urine by the kidneys. This route of cortisol clearance has little bearing on overall cortisol clearance rates or circulating levels. Nevertheless, it is significant in clinical practise as 24-hour urinary free cortisol measurements are commonly used in the evaluation of suspected hypercortisolism. In CKD, the renal clearance of free cortisol and the absolute amount in a 24-hour urine sample are both reduced.[274, 291] This means that measurement of 24-hour urine free cortisol is not a reliable test for evaluating suspected hypercortisolism in the context of CKD.

1.7.1.5 Cortisol protein binding

Circulating levels of free cortisol are determined by the balance between adrenal secretion, levels of corticosteroid-binding globulin (CBGs) and systemic metabolism.[298] Protein binding of cortisol to CBG is unchanged in CKD. CBG concentrations are comparable across CKD stages and health, cortisol binding affinity to CBG is similar in heathy and uraemic serum, and the total apparent distribution volume for cortisol is equivalent in CKD and health.[187, 297, 302] This means systemic measures of cortisol can generally be compared between CKD and control cohorts without confounding by shifts in the unbound fraction of cortisol available for signal transduction.

1.7.1.6 Impact of dialysis on cortisol

In patients with end-stage renal failure, the life-sustaining treatment of regular dialysis can itself influence cortisol levels in addition to disease processes. Cortisol is removed from the circulation during haemodialysis.[303, 304] However, this does not necessarily lead to a reduction in circulating cortisol levels. While a drop in cortisol levels from beginning to end of a haemodialysis session has been described in some studies [272, 275], other studies have reported a rise, implying increased cortisol secretory activity during dialysis.[188, 281] The discrepant findings could be due to differences in patient populations or administration of dialysis. Peritoneal dialysis also removes cortisol from the circulation, but does not restore normal cortisol levels compared to healthy controls.[305] Besides these acute effects, there is limited knowledge about the impact of chronic dialysis on long-term regulation of cortisol. No significant difference in morning cortisol levels were observed between patients with

advanced pre-dialysis kidney failure and haemodialysis-treated patients in several studies, but group sizes were small.[274, 285, 289] Further studies report no significant difference in morning cortisol levels between haemodialysis-treated and peritoneal dialysis-treated patients, but again small group sizes limit robust conclusions.[279, 283] Without data on systemic cortisol levels from larger cohorts and data on diurnal cortisol levels, potential effects of long-term dialysis or dialysis modality on cortisol levels cannot be reliably evaluated.

1.7.2 HPA axis regulation of cortisol secretion in CKD

Circulating levels of cortisol and diurnal rhythm are determined by the finely tuned balance between adrenal secretion of cortisol under hypothalamic-pituitary control and its metabolism prior to urinary excretion. In this section we will consider research relating to the dysregulation of the HPA axis that could underpin excess cortisol exposure in CKD.

Administration of synthetic ACTH followed by measurement of serum cortisol is a method utilised to examine one aspect of HPA function and adrenal capacity to secrete cortisol. Adrenal responsiveness to ACTH is generally preserved in people with CKD, with multiple studies reporting comparable cortisol responses [274, 279, 282, 306] and one study reporting higher peak cortisol levels in renal impairment relative to control groups.[285] Close examination of findings in some of these studies may point to more sustained elevation of circulating cortisol following ACTH administration in CKD, but confirming this requires further time-course analyses.[282, 285] In contrast, insufficient cortisol responses to ACTH have been reported in specific subgroups with CKD.[277, 307-309] However, participant selection in these studies included biases for adrenal insufficiency (e.g. referral for adrenal insufficiency testing,

history of glucocorticoid-treated glomerular disease, persistent hypotension or amyloid disease) that hamper their generalisability. Consequently, unless other risk factors for adrenal insufficiency are present, ACTH-induced cortisol release appears intact in CKD without notable differences to healthy populations.

Examinations of endogenous ACTH levels offer some further insight into HPA axis regulation at the hypothalamic-pituitary level. Levels of ACTH are variably reported as elevated or not significantly different in CKD compared to control groups. However, many of these studies rely on one-off measurements of early morning ACTH in blood. Evaluation of endogenous ACTH levels entails similar challenges as for cortisol levels, in that levels are highly dynamic over 24 hours, with many studies relying on relatively small sample sizes and significant heterogeneity in study populations. Acknowledging these limitations, comparisons of early morning ACTH level between dialysis-treated patients and control groups have found significant increases [274, 279, 284], trends for non-significant increases [278, 282] or no differences [271, 273, 281] (Table 1.6). Studies involving patients with pre-dialysis CKD tended to be larger but give a similarly mixed picture. One study involving participants from all stages of CKD reported increasing morning ACTH with loss of kidney function.[274] Other studies involving participants with mild to moderate CKD and other comorbidities (severe obesity, type 2 diabetes or adrenal adenoma) identified no correlation of ACTH levels with eGFR.[269, 286, 288] Diurnal rhythm of ACTH remains generally recognisable in advanced CKD, although nocturnal ACTH levels appear elevated compared to healthy volunteers according to two small studies.[271, 273] Overall, a trend emerges from the reviewed literature that elevations in ACTH are more readily observed in cohorts with advanced CKD, and less evident in early stages

Table 1.6: ACTH levels in CKD

Citation	Study Population	n	Findings
Studies reporting higher ACTH in CKD			
Raff et al. (2013)	Patients on Dialysis and Healthy Controls	24	high midnight ACTH in dialysis group ($p<0.05$)
Deshmukh et al. (2005)	Patients with CKD and Healthy Controls	11	high evening ACTH in CKD group ($p=0.04$)
Oguz et al. (2003)	Male Patients with CKD	60	high ACTH in CKD groups ($p<0.001$)
van Herle et al. (1998)	Patients on Haemodialysis and Healthy Controls	11	high ACTH in dialysis group ($p=0.02$)
Clodi et al. (1998)	Patients on Dialysis and Healthy Controls	21	high ACTH in dialysis groups ($p<0.05$)
Studies reporting no significant difference			
Vitolo et al. (2016)	Patients with Obesity (BMI>40 & normal creatinine)	50	n.s.
Asao et al. (2016)	Patients with Type 2 Diabetes (eGFR >15)	77	n.s.
Olsen et al. (2015)	Patients with Adrenal Adenoma (eGFR >30)	164	n.s. (Note: age mismatch between groups)
Jenkins et al. (1999)	Patients on Dialysis and Healthy Controls	24	trend for high ACTH in dialysis groups (p-value n.r.)
Letizia et al. (1996)	Patients on Dialysis and Healthy Controls	34	n.s.
Vigna et al. (1995)	Patients on Dialysis for >10 years & Healthy Controls	17	n.s. with trend for high ACTH in dialysis groups ($p=0.06$)

Abbreviations: n.s. – no significant difference; BMI – body mass index; eGFR – estimated glomerular filtration rate; ACTH – adrenocorticotrophic hormone.

of CKD. Consistent with this notion, metabolic acidosis, low-grade inflammation and heightened stress related to life with a chronic disease are putative drivers for increased pituitary secretion of ACTH and manifest as complications of advanced CKD.[55, 253, 254, 310] The heterogeneous distribution of inflammation and acidosis among populations with CKD may also account for observed heterogeneity in reported findings on ACTH.

The dexamethasone suppression test is classically used to examine the negative feedback mechanism in the HPA axis, meaning the capacity to shut down adrenal cortisol secretion.

Cortisol levels in CKD exhibit partial resistance to suppression by dexamethasone (a potent glucocorticoid receptor agonist with minimal mineralocorticoid activity). Evidence is robust that the standard dose of 1mg Dexamethasone given orally at midnight is less effective at supressing morning cortisol in CKD. Consistent findings are reported by numerous studies [269, 284, 288, 296, 297, 311], while only one study indicated no significant difference to the healthy control group.[274] The pharmacokinetics of oral Dexamethasone appear unaltered in CKD, meaning the data reflect a genuine difference in endogenous cortisol physiology rather than an artefact related to effective Dexamethasone dose.[297] When higher doses or extended courses of dexamethasone are used, a similar level of cortisol suppression can be achieved in CKD as in control groups.[297, 312] This observation suggests lower hypothalamic-pituitary sensitivity to negative feedback regulation, which can be overcome at higher glucocorticoid stimulation. Another potential explanation is prolonged half-life of cortisol in CKD. Persistence of higher levels of cortisol in circulation could contribute to apparent dexamethasone resistance. Measurements of ACTH levels following dexamethasone would allow evaluation of hypothalamic-pituitary responsiveness to glucocorticoid suppression directly, but none of the available reports included such data.

1.7.3 Half-life, clearance and 11βHSD metabolism of cortisol in CKD

Direct evidence for prolonged half-life of circulating cortisol is available from two studies in patients with advanced CKD (Kawai et al.: $t_{1/2}$ = 2.9h vs. 2.1h & Wallace et al.: $t_{1/2}$ = 1.9h vs. 0.9h for CKD and control groups).[187, 188] The kidneys are important for the clearance of cortisol in two distinct ways. These are removal of cortisol metabolites in the urine following primarily hepatic metabolism and reversible inactivation of cortisol to cortisone via the

enzyme 11beta-hydroxysteroid dehydrogenase type 2 (11βHSD2) (Figure 1.2).[181, 313] Several studies have examined these facets of cortisol metabolism as drivers of increased cortisol half-life in CKD.

The cortisol metabolites THF, 5α-THF and THE accumulate in patients with impaired renal function. Serum levels for these metabolites increase up to nine-fold in patients with CKD compared to control groups.[275] At the same time, the total amount of THF, 5α-THF and THE in 24-hour urine samples is reportedly similar in people with moderate CKD and healthy people (see section 1.7.1.4). This means that renal clearance of THF, 5α-THF and THE is reduced in CKD, as would be expected with a reduction in renal solute clearance in general. Little research has directly investigated hepatic metabolism of glucocorticoids in CKD. A study by Deck et al. provides some evidence that the accumulation of THF, 5α-THF and THE (the end products of hepatic cortisol metabolism) may attenuate hepatic inactivation and metabolic clearance of cortisol.[304] Clearance of cortisol in patients with end-stage renal failure was higher during dialysis than after dialysis, but the dialytic removal of cortisol itself accounted only for 20-35% of the difference. This suggests that dialytic removal of cortisol metabolites may enhance cortisol metabolism, through decreased inhibition by the end products of metabolism. Hence, hepatic metabolism of cortisol may be less effective in CKD.

11βHSD enzymes mediate the reversible interconversion between active cortisol and inactive cortisone (Figure 1.2).[189] 11βHSD1 (cortisol activating) and 11βHSD2 (cortisol inactivating) modulate tissue-specific cortisol exposure and influence circulating cortisol half-life.[196, 198] Peripheral 11βHSD metabolism of cortisol is altered in CKD, shifting the equilibrium towards

cortisol activation. On a systemic level, the balance of cortisol/cortisone interconversion by the 11βHSD enzymes can be measured as the ratio of cortisol to cortisone, or the ratio of their respective metabolites (THF & 5αTHF to THE). This cortisol/cortisone ratio is significantly elevated in serum from patients with end stage kidney failure compared to healthy controls [275, 276, 280] (Table 1.7). Furthermore, the cortisol/cortisone ratio in urine, or the ratio of their respective metabolites, is negatively correlated with kidney function in pre-dialysis CKD.[300, 301, 314, 315] These observations demonstrate a shift towards increased serum cortisol in the equilibrium of cortisol/cortisone interconversion as renal function diminishes. On a systemic level, this contributes to a prolongation of half-life of cortisol in circulation.

Reduction of 11βHSD type 2 activity is a determining factor for the altered cortisol/cortisone balance. Studies have shown that the kidneys are the major site for 11βHSD type 2 expression and activity. Accordingly, cortisone production is almost abolished in anephric patients.[313] Furthermore, the expression of 11βHSD2 in kidney biopsy tissue correlates negatively with kidney function.[301] This provides sound evidence that renal inactivation of cortisol to cortisone by 11βHSD2 is impaired in CKD.

The contribution of 11βHSD type 1 to altered cortisol/cortisone balance in chronic renal impairment is less clear. No direct measurements of 11βHSD type 1 activity or expression in relation to impaired renal function are available in humans. It is still worth considering that elevation of 11βHSD1 activity may occur in CKD. Inflammatory cytokines like tumour necrosis factor-α (TNFα) are elevated in CKD and are known to upregulate 11βHSD1 activity. In line with this hypothesis, the urinary (THF+5αTHF)/THE ratio – a surrogate marker of peripheral

Table 1.7: Surrogate markers of cortisol metabolism by 11βHSD enzymes

Citation	Study Population	n total	Groups	n per group	Age	Male	Sample	(THF+5αTHF)/THE ratio	F/E ratio	Finding
Sagmeister et al. (2019)	Patients with CKD (stage 0-5)	342	n/a		63 [50-75] (median; IQR)	61%	spot urine	Spearman ρ= -0.116 (p=0.032) [correlation with eGFR]		urine THFs/THE increases with renal impairment
Gant et al. (2018)	Patients with Type 2 Diabetes (CKD stage 0-5)	373	n/a		64±9 (mean±s.d.)	58%	24h urine	std. β= -0.36 (p<0.001) [correlation with eGFR]	std. β= -0.17 (p<0.001) [for correlation with eGFR]	urine THFs/THE and F/E increase with renal impairment; significant in multivariable regression
Hammer et al. (2010)	Patients with CKD (stage 2-3)	112	n/a		54±12 (mean±s.d.)	49%	24h urine	no correlation with eGFR (p= n.r.)	no correlation with eGFR (p= n.r.)	n.s.
Henschkowski et al. (2008)	Patients with hypertension (CKD stage 0-4)	163	n/a		49.6 [18-84] (mean; range)	57%	24h urine	Pearson r= -0.26 (p<0.001) [correlation with CrCl]	Pearson r= -0.32 (p<0.001) [correlation with CrCl]	urine THFs/THE and F/E increase with renal impairment; significant in multivariable regression
Quinkler et al. (2005)	Patients with CKD undergoing kidney biospy (stage 1-5)	95	n/a		49.3 [18-83] (mean; range)	56%	24h urine	Pearson r= -0.406 (p<0.001) [correlation with CrCl]	Pearson r= -0.278 (p<0.01) [correlation with CrCl]	urine THFs/THE and F/E increase with renal impairment
N'Gankam et al. (2002)	Patients on Dialysis and Healthy Controls	97	CKD stage 5 (HD)	63	68 [30-91] (median; IQR)	65%	plasma	3.0±1.2 (mean±s.d.)	9.3±5.3 (mean±s.d.)	higher plasma THFs/THE and F/E in HD group (p<0.001)
			Healthy Controls	34	54 [24-78]	59%		1.5±0.4	4.5±2.3	
Homma et al. (2001)	Patients on Dialysis, Patients with Diabetes and Healthy Controls	149	Healthy Controls	69	29.6±12.1 (mean±s.d.)	55%	serum		4.0 (mean)	higher serum F/E in HD group than in control or DM groups (p<0.01)
			Diabetes Mellitus	44	57.2±11.6	77%			5.0	
			CKD stage 5 (HD)	36	61.6±11.2	58%			11.6	
Morineau et al. (1997)	Patients on Dialysis and Healthy Controls	60	Healthy Controls	50	n.r.	n.r.	serum & saliva		9.8±3.5 (mean±s.d.) 0.5±0.19 (mean±s.d.)	higher serum F/E in HD group (p<0.01);
			CKD stage 5 (HD)	10					23.5±3.9 *[serum]* 0.38±0.11 *[saliva]*	lower saliva F/E in HD group (p<0.01)

Abbreviations: HD – haemodialysis; IQR – interquartile range; s.d. – standard deviation; n.r. – not reported; std. – standardised; CrCl – creatinine clearance; eGFR – estimated glomerular filtration rate; THF – tetrahydrocortisol; THE – tetrahydrocortisone; F – cortisol; E – cortisone; n.s. – no significant difference; DM – diabetes mellitus.

cortisol activation – is independently correlated with the systemic inflammation marker C-reactive protein (CRP) in patients with CKD.[314] Additionally, putative restoration of 11βHSD type 2 activity through renal transplantation does not normalise the plasma cortisol/cortisone ratio or urinary ratio of THF(s)/THE.[316] It is therefore possible that upregulation of 11βHSD1 activity may occur in CKD, but further proof is required to confirm this hypothesis.

None of the studies that have examined cortisol/cortisone interconversion in CKD have considered a potential role of the co-factors NAD^+ or NADPH for shifts in 11βHSD enzymatic activity. CKD is generally associated with elevated oxidative stress, which is itself a driver of renal fibrosis and cardiovascular morbidity.[317, 318] Endogenous changes in CKD including upregulation of NADPH oxidases would be expected to reduce the cellular pool of NADPH. However, the observed changes in CKD favouring peripheral glucocorticoid activation indicate lower oxidative 11βHSD activity (type 2 mediated conversion of cortisol to cortisone) and possibly higher reductive 11βHSD activity (type 1 mediated conversion of cortisone to cortisol), going against the trend of the pro-oxidative milieu in CKD. Therefore, tissue- and cellular compartment-specific redox status, besides changes in 11βHSD type 1 or 2 expression levels, may be more important for cortisol/cortisone balance in CKD than systemic oxidative stress. Further research is required to address this question in greater detail.

In summary, the range of changes in cortisol metabolism and renal clearance in CKD synergistically cause prolongation of half-life and reduced clearance of cortisol from the circulation. Irreversible metabolic inactivation in the liver appears impeded by the accumulation of metabolic end-products, which in turn is a consequence of reduced renal

filtration of these metabolites. Additionally, reversible inactivation of cortisol by 11βHSD type 2 in the kidney is impaired. Finally, preliminary findings propose that peripheral activation of cortisol by 11βHSD type 1 could be upregulated in CKD, although further validation is necessary. The discussed shifts in 11βHSD enzyme activities both result in longer half-life of cortisol in circulation. Therefore, multiple mechanisms together account for the prolonged half-life of cortisol in CKD.

1.7.4 Summary of changes contributing to cortisol excess in CKD

Review of the literature reveals a pattern of subtle cortisol excess in CKD. This is not readily apparent from the body of evidence on morning cortisol measurements alone. Morning cortisol levels are variably reported as reduced, normal or elevated in CKD. Certain studies with larger study cohorts and arguably more robust methodology have reported negative correlations of morning cortisol with eGFR, especially in populations with additional comorbidities. Nevertheless, there are considerable methodological and conceptual limitations with morning cortisol as a marker of cortisol exposure. Importantly, further evidence supports the presence of cortisol excess in CKD. Differences to normal physiology are much more apparent in settings where cortisol is expected to be suppressed. Measurements of cortisol levels at the evening nadir or serially over 24 hours reliably demonstrate reduced rates of cortisol decline, resulting in higher evening-time cortisol levels. Furthermore, studies consistently show impaired suppression of cortisol following dexamethasone. These are hallmarks of elevated cortisol exposure in CKD.

The underlying causes for cortisol excess in CKD are multifactorial, and their relative contribution is likely to vary depending on severity of CKD and co-existing morbidities (Figure 1.5). One important explanation for the observed changes is reduced clearance of cortisol. Cortisol half-life is prolonged as a result of synergistic changes directly related to renal impairment. Reduced renal filtration of cortisol metabolites and their systemic accumulation appears to impede enzymatic clearance of cortisol in the liver. Additionally, loss of 11βHSD type 2 expression in the kidney reduces the reversible inactivation of cortisol. Dialysis treatments are not capable of fully reversing these changes.

Alongside the changes in cortisol clearance, there are probably co-existing changes in HPA axis regulation. At first glance, cumulative adrenal cortisol secretion over 24 hours appears normal in mild to moderate CKD, based on cortisol metabolite content in 24-hour urine samples. However in the context of prolonged half-life for cortisol, this observation signifies inadequate negative feedback to downregulate adrenal cortisol output and maintain healthy systemic cortisol levels. Deficiencies in negative feedback mechanisms in CKD are also supported by data from dexamethasone suppression tests. Finally, there are reports that baseline pituitary ACTH secretion is elevated in some CKD cohorts, albeit not all. Inflammation and metabolic acidosis upregulate the HPA axis and could account for variable HPA axis activation among people with advanced CKD.

All these subtle changes in endogenous cortisol regulation promote chronic cortisol excess in CKD. The pattern and chronicity of cortisol dysregulation are reminiscent of other conditions of mild cortisol excess. Even though the relative elevation of cortisol may appear modest,

sustained mild cortisol excess is increasingly recognised as a risk factor for morbidity and mortality.

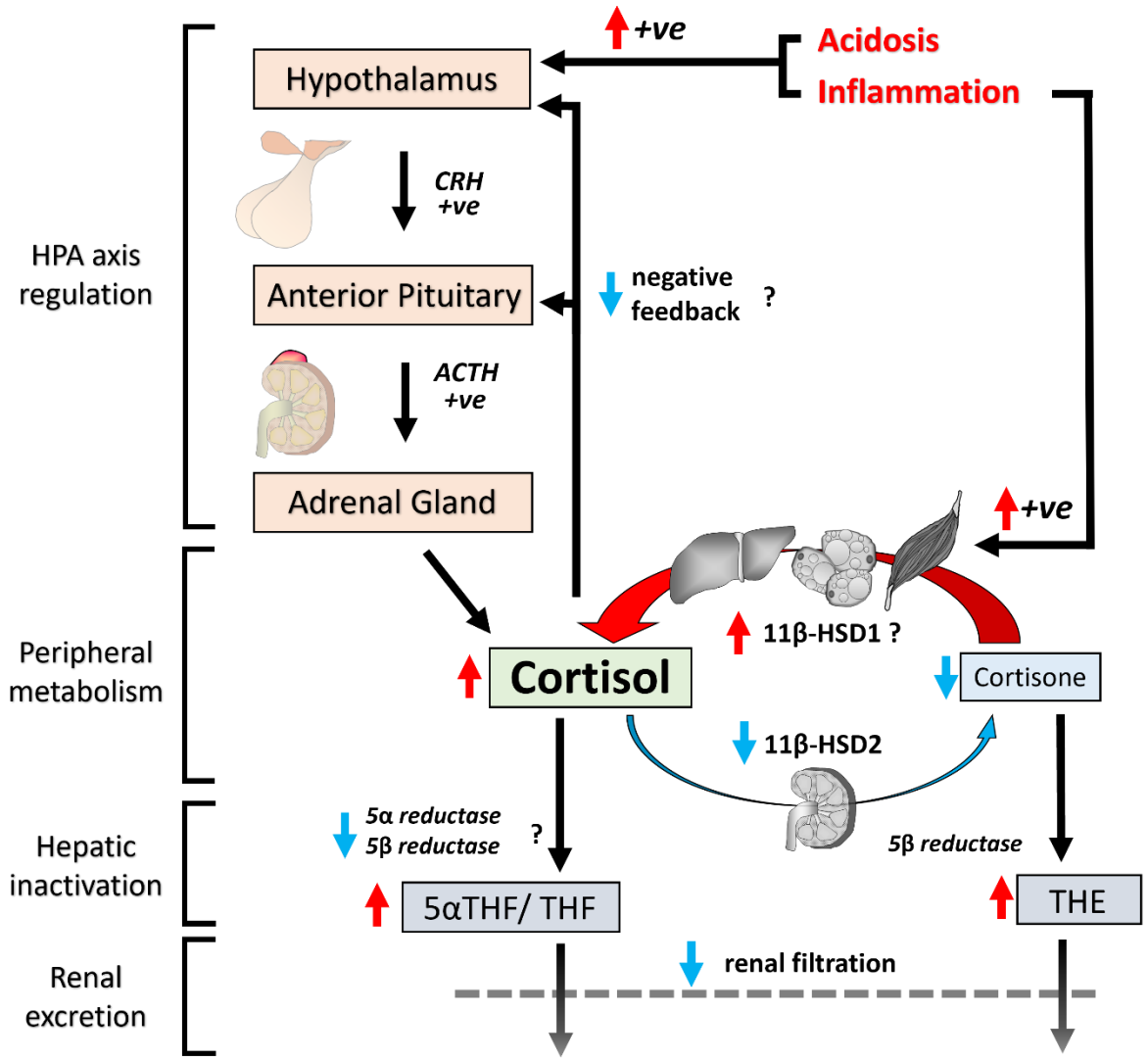


Figure 1.5 Schematic representation of changes in cortisol regulation in CKD.
Cumulative exposure to circulating cortisol increases in CKD for several reasons. Clearance pathways for cortisol are impaired in CKD because reduced renal filtration causes accumulation of cortisol metabolites in circulation with postulated end-product inhibition of hepatic cortisol metabolism. Furthermore, loss of 11ϐHSD2 in the kidney reduces cortisol inactivation to cortisone, contributing to prolonged half-life of cortisol in circulation. Peripheral cortisol activation by 11ϐHSD1 is possibly upregulated at the same time due to increased levels of inflammatory cytokines. Inflammation and acidosis also have the capacity to induce HPA axis activation. Finally, negative feedback mechanisms in the HPA axis to suppress adrenal cortisol secretion appear to be less effective in CKD. Red arrows indicate upward changes, blue arrows indicate downward changes and question marks indicate postulated changes based on indirect evidence.

1.8 11βHSD1 as a therapeutic target against sarcopenia – rationale for hypothesis

The primary focus for this research project will be 11βHSD1 and its role in skeletal muscle in conditions of renal impairment. 11βHSD1 has already been introduced above as an enzyme that converts inactive cortisone (dehydrocorticosterone in rodents) to active cortisol (corticosterone in rodents) (see section 1.5.3). Several strong reasons support the investigation of 11βHSD1 as a potential therapeutic target within the endogenous glucocorticoid system for the indication of sarcopenia in CKD. Not least because it avoids certain challenges with intervening in the endogenous glucocorticoid system. Although there is evidence of sustained glucocorticoid excess in CKD, the inherent homeostatic properties of the HPA axis make it problematic to correct this abnormality through direct pharmaceutical interventions. It is pharmaceutically feasible to inhibit systemic glucocorticoids actions via blockage of adrenal synthesis or glucocorticoid receptor antagonism.[319] However, low levels of inhibition result in compensatory HPA axis upregulation, while high levels of inhibition can produce life-threatening glucocorticoid deficiency. These difficulties are illustrated by the observation that treatment with the glucocorticoid receptor antagonist RU-486 (mifepristone) fails to replicate the protective effects of adrenalectomy in rats with acidosis-induced muscle atrophy.[257, 258] Hence, a more targeted approach for modulating glucocorticoid signalling within skeletal muscle may hold greater promise to reverse sarcopenia-promoting metabolic dysregulation.

1.8.1 Evidence for 11βHSD1 changes in CKD

Human studies investigating changes in 11βHSD1 activity in CKD have already been discussed in section 1.7.3 above. In brief, there is indirect evidence based on urinary cortisol metabolite ratios that systemic glucocorticoid activation by 11βHSD1 is elevated in CKD and that systemic inflammation is the strongest independent predictor for these changes.[320] This is biologically plausible, given that inflammatory cytokines such as TNFα are elevated in CKD and upregulate 11βHSD1 activity. Likewise, 11βHSD1 upregulation has been demonstrated in skeletal muscle and other tissues with inflammation in humans.[321-323] However, no direct evidence or tissue-specific analysis of 11βHSD1 changes in human CKD is available to date.

Additional information on 11βHSD1 changes in conditions of renal impairment is available from preclinical studies. Uraemic serum treatment increased 11βHSD1 expression *in vitro* in primary human hepatocytes.[324] Two studies report *in vivo* data from rodent models with renal impairment. Lauterburg et al. describe reduced 11βHSD1 mRNA expression but unchanged protein expression in liver from rats following uninephrectomy.[325] Metabolite ratios of glucocorticoids as an indirect marker of 11βHSD1 activity seem to indicate reduced glucocorticoid activation following uninephrectomy. However, the interpretation of these findings is problematic, as uninephrectomy is not a recognised model of chronic renal impairment. Indeed, data from the same study indicates only minimal changes in overall renal function and compensatory hyperfiltration with uninephrectomy. A more robust study was conducted by Chapagain et al., using the 5/6-nephrectomy model in rats as well as the adenine diet model in rats and mice to replicate persistent uraemia conditions *in vivo*.[259] This study found direct evidence of increased 11βHSD1 mRNA and protein expression in liver and adipose

tissue, as well as increased 11βHSD1 activity in liver. There was no evidence of elevated 11βHSD1 mRNA expression in skeletal muscle, but 11βHSD1 activity in muscle was not assessed. Elevations in 11βHSD1 activity and changes in tissue-specific glucocorticoid exposure occurred in the absence of changes in circulating corticosterone levels, highlighting the relevance of 11βHSD1 for autocrine/paracrine signalling. To conclude, the preclinical evidence to date is supportive of preliminary evidence from human studies that 11βHSD1 activity is upregulated in CKD [259, 320], but further research is necessary to test this hypothesis in a robust fashion.

1.8.2 Evidence for 11βHSD1 in the pathophysiology of sarcopenia

Tissue-resident glucocorticoid activation by 11βHSD1 is critical for metabolic responses in skeletal muscle. Cell culture and animal experiments have been key for understanding the role of 11βHSD1. As shown *in vitro*, muscle cells are equipped with an autonomous signalling machinery that allows 11βHSD1 to moderate insulin signalling, nutrient utilisation and intracellular protein turnover.[326, 327] *In vivo*, suppression of 11βHSD1 activity prevents the manifestation of insulin resistance in multiple models of metabolic stress, including in models of uraemia.[182, 259, 328] This finding is significant, as insulin resistance is a major component of the muscle wasting phenotype in CKD. Animal studies also demonstrated a direct involvement of 11βHSD1 in muscle wasting. Genetic deletion of 11βHSD1 protects against the muscle wasting effects of glucocorticoids in mice, even when the active form of corticosterone is administered.[329] Evidence from preclinical research thus characterises 11βHSD1 as a mediator of metabolic dysregulation and atrophy in skeletal muscle.

The preclinical data is supplemented by studies in humans that corroborate associations between 11βHSD1 and markers of sarcopenia. Convincing data from muscle biopsies and *in vivo* metabolic studies confirms that 11βHSD1 is expressed in human skeletal muscle and has cortisol-producing activity.[190, 263, 330] Two observational studies have examined 11βHSD1 specifically in relation to age-related loss of muscle mass and strength. Kilgour et al. found that skeletal muscle 11βHSD1 mRNA expression correlated negatively with maximum voluntary isometric knee extensor strength after adjustment for gender and height.[331] 11βHSD1 expression in skeletal muscle did not correlate with mid-thigh quadriceps cross-sectional area measured by MRI. Other glucocorticoid measures including plasma cortisol, total urinary glucocorticoids or urinary (THF+5αTHF)/THE ratio were not correlated with muscle strength or size, albeit samples size was relatively small in this study. In the second study by Hassan-Smith et al., skeletal muscle 11βHSD1 mRNA expression was also correlated negatively with muscle strength, measure as handgrip strength.[193] Total lean mass measured by DEXA was associated with muscle 11βHSD1 expression in male participants, but not in females. Again, urinary (THF+5αTHF)/THE ratio was less useful as a predictor of muscle strength than muscle 11βHSD1 expression. These observations are consistent with 11βHSD1 in skeletal muscle as a determinant of muscle strength, although the associative character of this data does not prove causal links.

1.8.3 Evidence for 11βHSD1 inhibition as a potential therapy for sarcopenia

Additional features make 11βHSD1 an attractive target for therapeutic intervention in sarcopenia, besides its putative pathophysiological role. As mentioned earlier, animal studies have already delivered proof-of-concept that genetic elimination of 11βHSD1 prevents loss of

muscle strength and mass in glucocorticoid excess conditions *in vivo*.[329] The same effect of protection against myopathy has been observed in a rare case report of an individual with Cushing's Disease and genetic defect in 11βHSD1 activity.[332] This constitutes the earliest clue that 11βHSD1 inhibition may be efficacious against muscle loss and weakness in humans.

A sizeable arsenal of pharmaceutical small molecule inhibitors of 11βHSD1 has been developed over the past two decades. This was driven especially by an interest in 11βHSD1 inhibition to treat metabolic syndrome, type 2 diabetes and non-alcoholic fatty liver disease. Indeed, phase II clinical studies have demonstrated beneficial effects with 11βHSD1 inhibitors for these indications.[265, 333, 334] Advancement of these agents to further clinical trials for these indications has since been suspended, as treatment effects were only modest in magnitude. Still, a range of highly selective and potent 11βHSD1 inhibitors are now available as a consequence of these efforts.[335] Detailed safety profiles have already been established for these agents in multiple clinical populations, with generally very good tolerability and few adverse effects.[336-338] The development expenditure and risk with exploring 11βHSD1 inhibition for novel indications such as sarcopenia is therefore considerably lower than with therapeutic targets that have not yet been developed to this level. Hence, there is a clear and apparent route for clinical translation and further development of 11βHSD1 inhibition as a treatment for sarcopenia.

Finally, exploratory data from two Phase II studies offer direct evidence that 11βHSD1 inhibitors are capable of improving skeletal muscle phenotype. The first study tested the 11βHSD1 inhibitor S-707106 in 16 patients with inoperable or recurrent Cushing's syndrome

over 24 weeks.[339] This intervention led to an improvement in body muscle percentage assessed by bioelectrical impedance analysis. Limitations with this study include its open-label design and lack of an appropriate control group. A second study tested the 11βHSD1 inhibitor AZD4017 in 31 overweight women with idiopathic intracranial hypertension over 12 weeks.[340] The design for this study was a double-blind, placebo-controlled trial. The intervention improved total lean body mass assessed by DEXA in the treatment group, and no improvement was observed in the control group. While these exploratory data need to be interpreted with caution, they strengthen the rationale to evaluate 11βHSD1 inhibition as a therapeutic strategy against sarcopenia.

1.9 Hypothesis and Aims

Reviewing the literature leads to a conclusion that involvement of 11βHSD1 in the pathophysiology of muscle wasting in CKD is highly plausible, but specific and important gaps in knowledge remain. Firstly, several conditions associated with kidney disease depend on signalling via the glucocorticoid receptor as an obligatory co-factor to induce atrophy pathways in skeletal muscle. However, the reason for this dual requirement of glucocorticoid signalling and other atrophic stimuli is not clear. Secondly, CKD is associated with shifts in glucocorticoid metabolism that point towards mild chronic glucocorticoid excess with potentially elevated 11βHSD1 activity. Yet, the precise causes, pattern and consequences of 11βHSD1 upregulation in CKD have not been defined. Thirdly, emerging data from human observational studies and clinical trials identify 11βHSD1 as a direct regulator of muscle size in humans. This poses the question whether 11βHSD1 could be targeted in CKD to prevent muscle wasting. These salient points have shaped the central hypothesis of my PhD project:

***Overactivity of 11βHSD1 in skeletal muscle contributes to muscle wasting in chronic kidney disease, and inhibition of 11βHSD1 is a novel therapy to prevent muscle wasting in kidney disease*.**

A range of complementary experimental approaches will be necessary to conduct a thorough and robust investigation of this hypothesis. An animal experiment will provide critical insights into therapeutic efficacy and systemic consequences of targeting 11βHSD1 to prevent muscle wasting in CKD. Muscle cell culture experiments will be used to elucidate signal pathway interactions between atrophic stimuli, 11βHSD1 regulation and intracellular protein turnover.

Finally, analysis of fresh skeletal muscle biopsies from donors with and without CKD will permit validation of pathophysiological changes in human disease. Mirroring these experimental approaches, the planned research project is structured into three work packages with the following aims:

1. Determine the therapeutic potential and accompanying physiological changes of 11βHSD1 inhibition to prevent muscle atrophy in a mouse model of persistent renal impairment.
2. Characterise the regulation of 11βHSD1 and its functional impact on myocellular protein metabolism under CKD-associated atrophic conditions in human primary muscle culture.
3. Assess variation in muscle 11βHSD1 activity in relation to kidney function, and explore 11βHSD1 activity as a risk factor for sarcopenia

Chapter II - Methods

Common laboratory reagents and consumables were purchased from Sigma Aldrich (UK), a subsidiary of Merck Life Science UK Limited, unless stated otherwise.

2.1 Methods: Animal experiment

2.1.1 Mouse model of renal impairment

Wild-type C57BL/6 mice were obtained from Charles River. Mice with global genetic deletion of 11βHSD1 were derived in-house from an established breeding colony on C57BL/6 background. The genetic and phenotypic status of prodigy from this colony has previously been described in detail.[263, 329, 341] Animals were housed in standard conditions with ad libitum access to food and water.

Animals started the experimental protocol at the age of 8-9 weeks. Male mice with wild-type and 11βHSD1-KO genotype were randomised to continue normal chow or receive chow supplemented with adenine (0.15-0.18% wt/wt) for 7 weeks. Adenine-supplemented chow was obtained from Special Diets Services (Augy, France) as a pre-mixed custom-manufactured research diet. Adenine diet supplementation has previously been validated as a specific and reliable methodology for modelling the phenotype of sustained renal impairment.[342-344] Body weights of animals were measured ≥3x per week for the duration of the experimental protocol. Animals with weight loss exceeding 20% relative to baseline were culled to avoid undue suffering in line with pre-specified welfare severity limits. At the end of the 7-week protocol, blood was collected by cardiac puncture under terminal anaesthesia and death confirmed by cervical dislocation.

Muscles and internal organs, including kidney, liver, spleen, visceral fat and heart, were dissected, weighed and immediately preserved for later analysis. For bilateral organs, the average weight was used for analysis. Tissues for mRNA analysis were snap-frozen in liquid nitrogen and stored at –80°C. Tissue for histology were fixed in 4% and stored in 70% ethanol. Tissues for 11βHSD1 activity assays were maintained in serum-free RPMI media at 4°C for 1-2 hours until testing. After allowing blood to clot at room temperature, serum was separated by centrifugation at 10,000rpm for 15 minutes and stored at -80°C until testing. Experiments complied with the Animal (Scientific Procedures) Act 1986 and received approval from the Birmingham Ethical Review Subcommittee (project license PP3501305).

The experiment was performed in male mice only. Previous studies have demonstrated that female mice exhibit relative resistance to extrarenal complications of reduced kidney function, including relative protection from skeletal muscle atrophy, bone remodelling and cardiac electrophysiological disturbances.[345-347] This sex-divergence is not restricted to the adenine diet model, but also evident in other CKD models such as the 5/6-nephrectomy model.[345] The phenomenon is postulated to reflect a protective role of oestrogen against the development of complications associated with CKD.[348-350] To derive maximal utility of the animal model for the stated research objective and to minimise use of experimental animals, a decision was reached to perform the animal experiment in male mice only.

A statistical power analysis was performed to estimate the required sample size for detecting skeletal muscle atrophy with the adenine model of renal impairment. This analysis considered how many animals would be required per group to detect a change in muscle weight with

adenine treatment using a 2-tailed independent t-test at significance level p=0.05 and power 0.9. Published data in the adenine-induced model of uraemia indicate muscle wasting of 20% with a coefficient of variation equal 0.1 for muscle weight measurements (Mandai 2018). Using GPower version 3.1.9.7, the calculated sample size per group is 7. An additional 25% margin was added to the calculated sample size to mitigate against potential animal attrition and variability in estimated effect size. Therefore, a minimum of 9 animals per group were included in the experiment.

A limited extension to the original experiment was carried out to provide additional kidney tissue samples for the direct measurement of 11βHSD1 oxoreductase and 11βHSD2 dehydrogenase activities. The extension experiment included 6 additional wild-type animals on normal chow and 4 additional wild-type animals on 0.15% adenine chow for a shortened duration of 5 weeks.

2.1.2 Grip strength testing in mice

Grip strength was measured at baseline, week 2, week 5 and at the end of the experimental protocol with a digital grip strength meter for mice (Linton Instrumentation, Leeds, UK). For each measurement, an animal was allowed to grip onto the metal plate with four paws, held with a pincer grip at its tail and drawn backwards until the grip released. The maximum of three readings per animal and session was used for analysis.

Baseline, week 2 and week 5 grip strength measurements were disrupted by access restrictions to the animal research facility during Covid-related social distancing. Longitudinal analysis of

grip strength is therefore limited to the subset of animals with complete datasets. Grip strength at the end of the experiment was measured for all animals.

2.1.3 Glucose tolerance test in mice

Glucose tolerance testing was performed during week 5 of the experimental protocol. After fasting animals from 8am for 5 hours, baseline glucose in blood was measured with a glucometer (Accu-Chek, Roche) from a small cut to the tail vein. An intraperitoneal injection of glucose (2mg/kg in sterile water) was administered to animals and repeat glucose measurements taken at 15min, 30min, 60min, 90min and 120min. Area under the curve data were calculated in GraphPad Prism version 9.5 for each individual animal with the fasting glucose level taken as the baseline.

2.1.4 Mouse serum analysis

Serum samples were sent to IDEXX Bioanalytics (Wetherby, UK) for quantification of creatinine, urea, alanine transferase, creatine kinase, total cholesterol and triglycerides. Further information on the analytical methodologies employed at IDEXX Bioanalytics are detailed in their Directory of Services 2023 available from https://www.idexxbioanalytics.com/directory-of-service-download-idexx-bioanalytics.

Corticosterone and IL-6 in mouse serum samples were analysed with commercially available ELISA kits according to manufacturer's instructions (corticosterone: product no. KGE009, R&D Systems Europe Ltd., Abingdon, UK (lower limit of detection: 0.028ng/ml; coefficient of

variation: ≤7.5%); mouse IL-6: product no. DY406-05, R&D Systems Europe Ltd., Abingdon, UK (assay range: 15.6 – 1,000 pg/ml; coefficient of variation: data not available)).

2.1.5 Histology of animal tissues

Formalin-fixed quadriceps muscle and kidneys were sent to IDEXX Bioanalytics (Columbia, USA) for paraffin embedding, sectioning and histological staining. Quadriceps samples were sectioned in the transverse plane across the mid-belly of the muscle to show the muscle fibres in cross-sectional orientation. Kidney samples were sectioned in the coronal plane across the middle of the organ to show the cortex, medulla and collecting system. Both tissue types were stained with haematoxylin and eosin. IDEXX Bioanalytics returned the stained slides for evaluation using an Olympus BX53 microscope.

Quantitative analysis of muscle fibre size was performed on 3 images (100x magnification) of the vastus medialis for six mice per group. Using ImageJ software, 100 measurements of muscle fibre cross-sectional area were taken from each image. Fibre cross-sectional area data were transformed with the square root function prior to further analysis.

2.1.6 Mouse tissue mRNA analysis

The methodology for RNA isolation and analysis has previously been described in the literature by our group.[351] It is outlined again here for ease of reference. To extract mRNA, 50-100mg mouse tissues were mechanically ruptured and lysed in TRIzol™ reagent (Thermo Fisher Scientific, Loughborough, UK). Chloroform was used for liquid phase separation, and

isopropanol was used for RNA precipitation. Following 3 washes with 75% ethanol, the RNA pellets were resuspended in RNase-free water and stored at -80°C.

Reverse transcription and real-time PCR analysis were performed in two separate steps. Reverse transcription of mRNA to cDNA used a commercially available kit according to manufacturer's instructions (MultiscribeTM, Thermo Fisher Scientific, Loughborough, UK). Real-time PCR analysis was performed on the ABI7500 system (Applied Biosystems, Warrington, UK) using TaqMan® Gene Expression Assays (Thermo Fisher Scientific, Loughborough, UK). The list of primer probes for investigated gene targets is reported in Appendix I. Assay reactions were run in duplicate, and contained 2X TaqMan PCR Mastermix (Thermo Fisher Scientific, Loughborough, UK), 200 nmol TaqMan probe and 25–50 ng cDNA. The abundance of specific mRNAs in a sample was normalised to the housekeeper gene GAPDH for muscle tissue and 18S for kidney tissue. For graphical illustrations, standardized expression values were derived with the formula:

$$mRNA\ expression = 2^{(Ct_{housekeeper\ gene} - Ct_{target\ gene})}$$

In text, differential mRNA expression between the experimental condition and control condition was expressed as:

$$fold\ change = \frac{mRNA\ expression_{experimental}}{mRNA\ expression_{control}} \qquad \text{(for upregulation)}$$

$$fold\ change = (-1) \times \frac{mRNA\ expression_{control}}{mRNA\ expression_{experimental}} \qquad \text{(for downregulation)}$$

2.1.7 Generation of radiolabelled ^{3}H-dehydrocorticosterone

Radiolabelled 11-dehydrocorticosterone (DHC), which is used in the 11βHSD1 oxoreductase activity assay, is not commercially available. Tritiated ^{3}H-DHC was synthesised from commercially available ^{3}H-corticosterone (Perkin Elmer, Beaconsfield, UK) according to a previously reported protocol.[352]

20µCi of ^{3}H-corticosterone, 250mg of murine placenta (which has very high 11βHSD2 expression) and nicotinamide adenine dinucleotide (final concentration 500mM) were incubated in 500 µL of 0.1 M potassium phosphate buffer (pH 7.4) at 37^{o}C for 18 hrs in 10ml glass tubes. Steroids were subsequently extracted by liquid phase separation using dichloromethane. After evaporation to dryness at 50^{o}C under blowing air, steroids were resuspended in 100µl of dichloromethane.

The steroids were applied onto silica cards for separation by thin-layer chromatography (TLC) using ethanol : chloroform (8:92) as the mobile phase. To aid identification and localisation of labelled ^{3}H-DHC, lanes containing ^{3}H-corticosterone and unlabelled corticosterone (1 mg/ml) and DHC (1 mg/ml) were run in parallel on the TLC plates. A Bioscan imaging detector (Bioscan, Washington, DC, USA) was used to detect and quantify radiolabelled steroids; ultraviolet light was used to detect concentrated spots of fluorescing unlabelled steroids. The spot containing ^{3}H-DHC was cut from the TLC plate and transferred into a fresh 10ml glass tube for elution in 500ul of ethanol overnight at 4^{o}C. The steroid solution was aspirated, cleaned from residual silica by centrifugation at 100g and transferred to a clean glass container.

To test the purity of ^{3}H-DHC contained within the ethanol, 1µl was run on a TLC plate in parallel with labelled corticosterone and unlabelled corticosterone and DHC. Based on the level of detected radioactivity, the volume of ethanol was altered either through dilution or concentration by evaporation under nitrogen in a sample concentrator to give approximately 100 cpm/µl. 5µL of this stock solution was used per murine 11βHSD1 oxoreductase assay reaction.

2.1.8 11βHSD1 activity assay

50-100mg of fresh animal tissues, 11-dehydrocorticosterone (DHC) (final concentration 100nM) and tracer amounts of tritiated DHC were incubated in 1mL serum-free RPMI medium at 37^{o}C for 3 hours. Steroids were extracted from reaction media by liquid phase separation using dichloromethane. Subsequently, steroids were separated by thin-layer chromatography with ethanol/chloroform (8:92) as the mobile phase. Thin-layer chromatography plates were analysed with a Bioscan imager (Bioscan, Washington, DC, USA) to obtain the relative proportions of unchanged DHC and converted corticosterone. Fractional conversion of steroids was normalised to wet tissue weights, and 11βHSD1 enzymatic activity was reported as corticosterone production rate in fmol per mg tissue per hour:

$$enzymatic\ activity = \frac{product\ radioactivity}{product\ radioactivity + substrate\ radioactivity} \times \frac{substrate\ concentration}{reaction\ volume\ \times tissue\ weight\ \times reaction\ time}$$

This protocol has previously been described and validated.[263, 351]

2.1.9 11βHSD2 activity assay

Measurement of 11βHSD2 dehydrogenase activity followed the same principle as the 11βHSD1 oxoreductase assay. Fresh tissues were incubated in serum-free RPMI medium containing 100nM corticosterone along with tracer amounts of tritiated corticosterone at 37^{o}C for 1 hour. Steroids were extracted into dichloromethane, separated by TLC and analysed with a Bioscan imager as described above. Fractional conversion of steroids was normalised to wet tissue weights, and 11βHSD2 enzymatic activity was reported as DHC production rate in fmol per mg tissue per hour (see formula in section 2.1.8).

2.1.10 Statistical analysis for the animal experiment

Data was analysed and illustrated using GraphPad Prism 9.5. Data in figures is displayed as mean ± 95% confidence interval, unless otherwise specified. Aggregate data from experimental groups are described in text or tables as means ± standard deviation, unless otherwise specified. Comparisons between groups are reported in text as difference in means, percentage change or fold change depending on the respective data, alongside the p value for the statistical test. Negative fold change was calculated as -1/relative difference. Statistical analyses used independent t-tests, one-way ANOVA with Tukey's post-hoc test, two-way ANOVA with Tukey's or Dunnett's post-hoc test or non-parametric equivalents as appropriate. The specific statistical test for each analysis is stated in the figure legends. P-values less than 0.05 were taken as statistically significant.

2.2 Methods: Cell culture experiments

2.2.1 Myotube cell culture

The methodology for primary human muscle cell culture followed a previously described and validated protocol.[353] Fresh muscle tissue was coarsely minced with a scalpel prior to digestion in trypsin-EDTA at 37°C for 15 minutes. Proliferation media (Ham's F10 media (N6013, Sigma), 100 µg/mL penicillin/streptomycin, 20% foetal bovine serum) was added to inactivate trypsin and the cell suspension was incubated in an uncoated flask for 20 minutes to select out fibroblasts by preferential adhesion. The cells in suspension were then transferred into 0.2% gelatine-coated flasks for culture of myoblasts. Media was renewed every two days. Myoblasts were passaged at 60-70% confluence and used for experiments between passage 2 and 4. To differentiate myoblasts into myotubes, proliferation media was replaced with differentiation media (Ham's F10 media (N6013, Sigma), 100 µg/mL penicillin/streptomycin, 6% horse serum (heat inactivated, Gibco)). Cells were maintained in differentiation media for a minimum of eight days before starting experiments.

Pharmaceutic agonists and inhibitors for experiments were prepared in differentiation media at the concentrations stated in Table 2.1, unless otherwise specified. Media containing human serum used Ham's F10 media with 100µg/ml penicillin/streptomycin as a base, without serum from any animal source. The pH in differentiation media was adjusted through addition of 1M HCl or 1M NaOH. After allowing media to equilibrate at 5% atmospheric CO2 and ensuring minimal contact with air, pH measurements were performed with an electrochemical pH meter (Fisher Scientific, Loughborough, UK).

Table 2.1 Agonists and inhibitors used in cell culture experiments

Agent	Final concentration	Supplier
TNFα	10ng/ml	Merck Life Science UK Limited, Gillingham, UK
glycyrrhetinic acid (GA)	5µM	
cycloheximide	50µg/ml	
Cortisol	100nM	
Cortisone	100nM	
MG132	10µM	Fisher Scientific, Loughborough, UK

Cell culture experiments were repeated three times at minimum. For experiments with highest priority for the research question, experimental repeats used distinct primary myotube lineages from separate muscle tissue donors to reduce bias from donor traits. Further details of experiments are described in the figure legends.

2.2.2 Human tissue and serum samples used in cell culture experiments

Human tissue and serum samples for use in cell culture experiments were obtained from existing clinical studies with ethical approval. Skeletal muscle tissue samples were donated by patients with osteoarthritis undergoing elective joint replacement therapy (NRES REC 16/SS/0172). Key characteristics of muscle tissue donors are summarised in Table 2.2.

Serum samples were donated by ambulatory patients with chronic kidney disease or healthy controls enrolled in the SONIC study (clinicaltrials.gov ID NCT02535052; NRES REC 15/WM/0057) [354], and ambulatory patients with non-alcoholic fatty liver disease or healthy controls taking part in a study on sarcopenia in chronic inflammatory disease (clinicaltrials.gov ID NCT04 734496; NRES REC 18/WM/0167).[355] Serum and urine biochemistry profiles for serum donors were obtained from automated clinical assays (Roche Hitachi 702 Analyser,

Basel, Switzerland). eGFR was derived from the serum creatinine-based CKD-EPI equation. Serum CRP was measured using the CRP High Sensitive ELISA (IBL, Hamburg, Germany).

Table 2.2 Characteristics of muscle tissue donors for primary myotube cultures

Study ID	Age/ Sex	Joint	BMI	Comorbidity
MFX380	67 M	Hip	35.7	diabetes mellitus type II , hyperlipidaemia, hypertension, polyarthrosis, fatty change of liver
MFX381	72 F	Hip	34.8	diabetes mellitus type II , hypertension, gastro-oesophageal reflux disease, polyarthrosis, urticaria
MFX386	57 M	Hip	38.5	obesity, diabetes, hypertension, asthma, polyarthrosis
MFX387	69 M	Hip	33.7	Diabetes (diet controlled), polyarthrosis, high cholesterol
MFX389	75 M	Knee	39.8	Diabetes mellitus type II, hyperlipidaemia, hypertension
MFX392	84 F	Hip	34.1	hysterectomy
MFX393	63 M	Hip	36.2	hypertension, gastro-oesophageal reflux disease, polyarthrosis
MFX395	65 F	Hip	24.2	hypertension, high cholesterol
MFX426	55 F	Hip	30.7	polyarthrosis, hypothyroid, high cholesterol, previous hip replacement and revision surgery
MFX429	84 F	Hip	30.5	polyarthrosis, hypertension, hyperlipidaemia, gastro oesophageal reflux, hyperplasia of prostate
MFX440	68 M	Hip	32.5	polyarthrosis, hypertension, hyperlipidaemia
MFX443	63 F	Hip	37.8	obesity, fibromyalgia, gastro-oesophageal reflux, irritable bowel syndrome, polyarthrosis
MFX452	67 F	Hip	23.2	recurrent depressive disorder, polyarthrosis, psoriasis
MFX455	76 F	Hip	22.3	polyarthrosis, guttate psoriasis
RHH63	78 F	Knee	24.4	polyarthrosis

2.2.3 Analysis on conditioned media from cell culture experiments

Glucose concentration in media was measured with a glucometer (Accu Chek, Roche). Human IL-6 concentration in media was measured with a commercially available ELISA kit according to manufacturer's instructions (Catalog Number 88-7066, Invitrogen, Thermo Fisher Scientific, Loughborough, UK).

2.2.4 Cell culture mRNA analysis

RNA from cultured cells was isolated with the innuPREP RNA Mini Kit (Analytik Jena, Cambridge) as per the manufacturer's instructions. Reverse transcription to generate cDNA and subsequent real-time PCR was performed as previously described in section 2.1.6. A list of primers used for analysis can be found in Appendix I. GAPDH was used as a housekeeper gene for normalisation of expression values. Gene expression data was expressed as described in section 2.1.6.

2.2.5 Microscopy for cell cultures

Plain microscopy images were acquired on a Leica DMIL Type 090-135.022 III/02 microscope. For myotube thickness measurements, ImageJ software was used to determine cellular width 25 times per image on phase contrast images at 200x magnification. For Trypan Blue viability staining, cells were washed 3x with PBS, stained with 0.2% Trypan Blue (Gibco, Fisher Scientific, Loughborough, UK) for 1 minute and fixed immediately with 4% paraformaldehyde for 30 minutes at room temperature. For immunofluorescence staining, cells were fixed with 2% paraformaldehyde for 30 minutes, permeabilised with 100% methanol for 10 minutes and

blocked with 5% goat serum for 30 minutes, washing with PBS between steps. Incubation with mouse anti-desmin primary antibody (MA5-13259, Thermo Fisher, diluted 1:200 in PBS and 1% bovine serum albumin) was overnight at 4°C and incubation with goat anti-mouse secondary antibody (R37120, Thermo Fisher, diluted 1:1000 in PBS) for 1 hour at room temperature, again washing with PBS between incubations. Finally, nuclei were stained by addition of DAPI (diluted 1:5000 in PBS) for 5 minutes. Immunofluorescence images were acquired on a Leica DMI6000 confocal microscope.

2.2.6 11βHSD1 activity assay

Media containing 100nM cortisone along with tracer amounts of tritiated cortisone (Perkin Elmer, Beaconsfield, UK) was added to cell cultures, maintaining experimental treatment conditions where applicable. After incubation for 6 hours at 37^{o}C, conditioned media containing the steroids was collected into glass tubes. Further processing was performed as described in section 2.1.8. Fractional conversion of steroids was normalised to total cellular protein content, and 11βHSD1 enzymatic activity was reported as cortisol production rate in pmol per mg protein per hour (see section 2.1.8).

2.2.7 Protein assay

Cells were washed with PBS three times prior to lysis in water (for 11βHSD1 assays). Alternatively, an aliquot of protein solubilised in 0.1M NaOH / 1% triton X-100 was used (for protein synthesis assays). Protein concentration in lysate was measured with the Bio-Rad Protein assay, which is based on the colorimetric Bradford method, adhering to the

manufacturer's protocol (Bio-Rad, Hercules, CA, USA). Protein concentrations were taken as the mean of duplicate measurements.

2.2.8 Protein synthesis assay

Protein synthesis was measured as the rate of radiolabelled tyrosine assimilation into intracellular protein, replicating a previously described protocol.[326] Cell cultures in 12 or 24 well plates were incubated in media with 200nM unlabelled tyrosine and 2µCi/mL of ^{3}H-tyrosine for 1 hour, maintaining experimental treatment conditions where applicable. The media was then removed and cells were washed 3x with serum-free media, before adding 10% TCA to lyse cells. The cell lysate was transferred to Eppendorf tubes and centrifuged at 13,000g for 10min. The resulting pellet containing precipitated protein was washed three times with 1ml 10% TCA, centrifuging the sample between washes to retain all precipitated protein. Following the third wash, the supernatant was discarded and the protein pellet resuspended in 0.1M NaOH with 1% Triton X-100. An aliquot of solubilised protein was set aside for measurement of protein concentration as described above (section 2.2.7). The remainder of the solubilised protein was transferred to scintillation tubes containing 4ml of scintillation fluid. Radioactivity of each sample was measured using a Packard TRI-CARB 1600 TR liquid scintillation analyser (Perkin-Elmer, Beaconsfield, UK). Detected radioactivity was normalised to protein concentration and protein synthesis rate is reported as incorporated radioactivity (CPM) per hour per microgram protein.

$$protein\ synthesis\ rate = \frac{radioactivity\ per\ sample\ volume}{protein\ concentration\ \times\ labelling\ time\ (1\ hour)}$$

2.2.9 Protein degradation assay

Protein degradation was measured as the rate of radiolabelled tyrosine release from prelabelled intracellular protein, replicating a previously described protocol.[326] Cell cultures in 12 or 24 well plates were incubated in regular differentiation media with 0.5µCi/ml of ^{3}H-tyrosine for 48 hours to pre-label cellular proteins. Cells were then washed three times before applying 500µL media containing respective experimental treatment conditions plus 2mM unlabelled tyrosine. Following 24h incubation at 37^{o}C, the conditioned media was collected into Eppendorf tubes and placed on ice. Cells were washed with ice-cold PBS three times before lysis in 0.5M NaOH with 0.1% Triton X-100. The cell lysate was transferred into scintillation tubes for subsequent quantification of radioactivity. 100µL of bovine serum albumin (10mg/ml) and 67µL 100% TCA were added successively to the Eppendorf tubes containing the media and vortexed. The mixture was placed on ice for 1h to allow protein precipitation. Samples were then centrifuged at 13,000g for 10min and the supernatant was transferred to scintillation tubes. The protein pellets were resuspended in tissue solubiliser (Solvable^{TM}, Perkin Elmer, Beaconsfield, UK) before ultimately transferring them to fresh scintillation tubes. Radioactivity in the three sets of scintillation tubes, representing the intracellular protein fraction, TCA-precipitated extracellular protein fraction and TCA-soluble extracellular amino acid fraction, was quantified using a Packard TRI-CARB 1600 TR liquid scintillation analyser (Perkin-Elmer). Percentage protein degradation over 24h can be derived from 100 times the TCA-soluble radioactivity in the medium divided by the TCA-soluble plus the TCA-insoluble radioactivity in the medium plus the radioactivity in the cell layer:

$$protein\ degradation\ rate =$$

$$\frac{radioactivity_{TCA-soluble} \times 100}{(radioactivity_{TCA-soluble} + radioactivity_{TCA-insoluble} + radioactivity_{cell\ layer}) \times time\ (24hours)}$$

Additionally, secretion of pre-labelled intracellular protein into culture media over 24h was calculated as 100 times the TCA-insoluble radioactivity in the medium divided by the TCA-soluble plus the TCA-insoluble radioactivity in the medium plus the radioactivity in the cell layer:

$$protein\ secretion\ rate =$$

$$\frac{radioactivity_{TCA-insoluble} \times 100}{(radioactivity_{TCA-soluble} + radioactivity_{TCA-insoluble} + radioactivity_{cell\ layer}) \times time\ (24hours)}$$

2.2.10 Statistical analysis for cell culture experiments

Data was analysed and illustrated using GraphPad Prism 9.5. Data in figures is displayed as mean ± 95% confidence interval, unless otherwise specified. Aggregate data from experiments are described in text or tables as means ± standard deviation, unless otherwise specified. Comparisons between groups are reported in text as difference in means, percentage change or fold change depending on the respective data, alongside the p value for the statistical test. Negative fold change was calculated as -1/relative difference. Statistical evaluations used independent 2-tailed t-tests, one-way or two-way ANOVA with post-hoc tests for multiple comparisons. Data with skewed distribution was log-transformed for analysis and illustration, or evaluated with non-parametric equivalent tests as appropriate. As a general principle, data from experiments that used genetically distinct primary myotubes for replicates were analysed with paired or repeated-measures tests to account for non-experimental variation relating to

muscle tissue donors' individual traits. The specific statistical test for each analysis is stated in the figure legends. P-values less than 0.05 were taken as statistically significant.

2.3 Methods: Clinical studies

2.3.1 Study participants for the Renal Impairment in Secondary Care (RIISC) study

2.3.1.1 Participants

Cohort 1 included 325 participants with CKD sampled from the Renal Impairment in Secondary Care (RIISC) observational study cohort. The RIISC study is a mature cohort study that has gathered detailed clinical, laboratory and lifestyle data with the aim of identifying risk factors for CKD progression, morbidity and mortality. The protocol for the RIISC study has previously been described in detail.[356]

The principal recruitment criteria for the RIISC study are repeated here for ease of reference. Participants in RIISC had CKD stage 3 with declining estimated glomerular filtration rate (eGFR) of $\geq$5 mL/min/1.73m^2/year or $\geq$10 mL/min/1.73m^2/5years or proteinuria with urinary albumin-creatinine ratio (ACR) $\geq$70 mg/ mmol, or CKD stage 4/5. Receiving renal replacement therapy or immunosuppressive treatment were exclusion criteria for recruitment to the RIISC study. A total number of 932 patients have been enrolled in the RIISC cohort at two teaching hospitals in Birmingham, United Kingdom, between 2010 and 2015.

A subset of 331 cases in the RIISC cohort has pre-existing data on steroids in urine samples. Urinary steroid metabolome analysis has been carried out as part of a previously published study.[320] The sampling procedure has been described already in the published manuscript and is summarised again here for ease of reference. From a total of 932 cases enrolled in the RIISC cohort, 299 cases with incomplete biochemical data sets and 25 cases receiving systemic

GC treatment were excluded prior to sampling. 333 cases of 608 eligible cases were sampled for urinary steroid metabolome analysis. A stratified random sampling procedure was employed to balance the proportions of cases with eGFR ≤15, 15 - 30, 30 - 45 and ≥45 mL/min/1.73m^2 and to avoid oversampling cases from a narrow band of low eGFR which were preferentially enrolled in the RIISC cohort.

The original analysis presented as part of this PhD thesis used data from 325 participants. EQ5 data was not available for 6 cases, precluding their inclusion for any analysis of interest. Cystatin C data was not available for 19 cases, meaning these cases could not be included in the respective part of the analysis.

Use of data and samples for this research falls under ethical approvals granted by the South Birmingham Local Research Ethics committee (reference 10/H1207/6). All participants provided written informed consent, and the study was conducted in accordance with the Declaration of Helsinki.

2.3.1.2 Data sources

The analysis used existing data from the RIISC cohort, and the methodology for data collection has previously been described in detail.[320, 356] In brief, study participants' demographic information, past medical history and concomitant medications was taken from medical records. Diabetes mellitus was defined by history of diet-controlled diabetes, treatment with anti-diabetic medications or HbA1c greater than 48 mmol/mol. The EQ-5D-3L questionnaire

was used to ascertain self-reported information about problems with usual daily activities or mobility.[357]

Laboratory measurements for serum creatinine, cystatin C and urine albumin-creatinine ratio were performed on an automated clinical biochemistry analyser (Roche Hitachi 702 Analyser, Basel, Switzerland). Spot urine samples were collected at morning clinic visits and stored at −80^{o}C until steroid metabolome analysis. CRP was measured using the Full Range C-Reactive Protein SpaPlus assay (The Binding Site, Birmingham, UK). Creatinine-based eGFR was derived with the CKD-EPI equation [358], cystatin C-based eGFR was derived with the CKD-EPI cystatin C equation.[359]

2.3.2 Study participants for the Steroid Metabolism and Muscle Loss in Chronic Kidney Disease (SMK) study

2.3.2.1 Participants

Cohort 2 included 17 patients with CKD and 14 healthy volunteers from the Steroid Hormone Metabolism and Muscle Loss in Chronic Kidney Disease (SMK) study. This is an original single-centre case-control study to characterise steroid metabolism in chronic kidney disease and its role for skeletal muscle dysfunction. Participants were recruited between August 2022 and April 2023. Patients with CKD were recruited from renal outpatients at University Hospitals Birmingham and healthy volunteers were recruited via news bulletins at the University of Birmingham and from the 1000 Elders cohort.[360] Inclusion criteria were age between 60 to 80 years, eGFR less than 30ml/min for more than 3 months for participants with CKD or no

history of kidney disease or other major chronic illness for healthy volunteers. Exclusion criteria were treatment with steroid medications, primary neurological or muscle disease that impairs muscle function, BMI <18 or >35kg/m^2, cancer diagnosis with the previous 5 years (except non-melanoma skin cancer), hospitalisation with an acute illness in the previous month or participation in another clinical trial with a treatment intervention with the previous four months. Participants attended the Clinical Research Facility at Queen Elizabeth Hospital at 8am following an overnight fast for a single visit to take part in the study.

Use of data and samples for this research falls under ethical approvals granted by the London – Stanmore Research Ethics committee (reference 22/PR/0360). The study has been registered in the ISRCTN registry (ISRCTN15497320). All participants provided written informed consent, and the study was conducted in accordance with the Declaration of Helsinki.

2.3.2.2 Data collection

Past medical history and medication history was recorded from electronic health records and confirmed with participants. Two questionnaires were administered to collect information about physical activity habits and malnutrition risk, the International Physical Activity Questionnaire – short form [361] and the Short Nutritional Assessment Questionnaire.[362] Data for serum creatinine, urine ACR, haemoglobin, total bicarbonate, HbA1c and serum albumin was taken from the latest available laboratory results on electronic health records. eGFR was calculated with the CKD-EPI creatinine equation.[363]

2.3.2.3 Skeletal muscle assessment

Measurements of height and weight were taken, and body mass index calculated as weight / height^2. Body composition was measured by bioimpedance analysis using a Tanita MC-780 P MA body-analyser. Appendicular muscle mass was calculated as the sum of estimated muscle mass in arms and legs. Appendicular muscle mass was normalised to body weight (expressed as %) or the square of body height (expressed as kg/m^2).

Forearm muscle strength was measured using a digital handheld dynamometer in the sitting position. After a demonstration and practise trial, 3 readings were taken on each side. The maximal reading from the dominant hand was used for analysis. Muscle function was assessed with the Short Physical Performance Battery, involving standing balance, gait speed and five-time repeated chair rises.[364]

2.3.2.4 Sample collection

Blood draws took place at 8am following an overnight fast. Blood samples were allowed to clot at room temperature for 1 hour, spun at 2,000g for 10min to separate serum and stored at -80^{o}C.

Skeletal muscle biopsies were taken from the vastus lateralis with the percutaneous Bergstrom technique.[365] After disinfecting and infiltrating local anaesthetic to the lateral thigh, a 5-10mm skin incision was made followed by sampling 100-200mg skeletal muscle tissue using a 5mm Bergstrom needle with applied suction. Incisions were closed with SteriStrips, a wound

dressing and a bandage. Muscle biopsy samples were maintained in serum-free Dulbecco's Modified Eagle's Medium (DMEM) at 4^{o}C for 1-2hours until 11βHSD1 activity testing.

Participants received a container together with verbal and written instruction on completing a 24-hour urine collection at home. 24-hour urine samples were returned within 24 hours of completing the collection. The samples were weight to determine total volume and stored as aliquots at -80^{o}C.

2.3.3 Steroid quantification in human urine and serum samples

Steroids were extracted and quantified using liquid chromatography – tandem mass spectrometry by the Steroid Metabolome Analysis Core, a specialist facility at University of Birmingham which specialises in multi-steroid analysis in biological samples. Serum was analysed through the validated method described here [340, 366-369]. Urine was analysed through the validated method described here [320, 370]. For additional information, a detailed description of the methods for steroid measurement is attached in Appendix II.

For 24-hour urine samples (SMK study), steroid concentrations were multiplied by urine volume to obtain the amount of steroid excreted in urine per day. Given that daily urinary steroid excretion cannot be determined for spot urine samples (RIISC study), only ratios of steroid concentrations were analysed for these samples. The ratio of cortisol metabolites (THF+5aTHF) to cortisone metabolites (THE) in urine has previously been shown to reflect the balance of systemic (i.e. total body) cortisol – cortisone interconversion by 11βHSD enzymes [371, 372], including for morning spot urine samples.[373, 374]

2.3.4 11βHSD1 activity assay

Measurement of 11βHSD1 enzymatic activity in quadriceps biopsies ex vivo followed the same principle as described in section 2.1.8. Fresh muscle tissue samples were incubated in 0.5mL serum-free DMEM containing 100 nmol/l cortisone along with tracer amounts of tritiated cortisone at 37^{o}C for 24 hours. Steroids in conditioned media were extracted using dichloromethane and separated by thin-layer chromatography with ethanol/chloroform (8:92) as the mobile phase. Thin-layer chromatography plates were analysed with a Bioscan imager (Bioscan, Washington, DC, USA) to obtain the relative proportions of unchanged cortisol and converted cortisol. Fractional conversion of steroids was normalised to wet tissue weights, and 11βHSD1 enzymatic activity was reported as cortisol production rate in fmol per mg tissue per hour. This protocol has previously been described and validated for human muscle biopsy samples.[330, 351] Assays were run in duplicate, if sufficient biopsy tissue was available.

2.3.5 Statistical analysis for clinical studies

GraphPad Prism version 9.5 was used for data analysis and illustration, except for multivariable regression modelling which was done using IBM SPSS Statistics for Windows version 29.0. Data in figures is displayed with mean ± 95% confidence interval, unless otherwise specified. Aggregate data are described in text or tables as means ± standard deviation, unless otherwise specified. Comparisons between groups are reported in text as difference in means or percentage change depending on the respective data, alongside the p value for the comparison. Statistical analyses used independent t-tests, Pearson correlation or one-way ANOVA with Tukey's post-hoc test for multiple comparisons. Data with skewed distribution was

log-transformed for analysis and illustration or evaluated with non-parametric equivalent tests as appropriate. The specific statistical test for each analysis is stated in the figure legends.

Variable selection for multivariable regression modelling was based on plausible associations with the outcome variable and statistical association in bivariate analysis. Where appropriate, the outcome variable for multiple linear regression was log-transformed to comply with the requisite statistical assumptions.[375] For binary logistic regression analysis, C-reactive protein as a continuous variable did not comply with the linearity assumption of independent variables and log odds.[376] The variable is therefore added into the model as a stratified variable (tertiles for C-reactive protein with limits ≤1.828µg/mL, 1.828µg/mL – 4.509µg/mL and >4.509µg/mL).

P-values less than 0.05 were taken as statistically significant, except in correlation matrices. In correlation matrices, statistical significance levels are reported as $\log_{10}$(p value) without prior adjustment of p values for multiple comparisons, but with declaration of the adjusted statistical significance level after accounting for multiple comparisons using the conservative Bonferroni method.

Chapter III – An in vivo experiment to assess 11βHSD1 suppression as a therapeutic approach for muscle atrophy in prolonged renal impairment

3.1 Introduction

3.1.1 Rationale for experimental approach

This first research results chapter discusses an animal experiment of blocking 11βHSD1 function in a mouse model of persistent renal impairment. In vivo studies using animal models present a valuable tool for investigating muscle atrophy in renal impairment.[377] Chronic kidney disease leads to systemic complications and interrelated multi-organ pathology.[46] Such multi-system disease processes can be replicated more faithfully in whole organism studies, instead of cell culture or tissue studies. Similar considerations apply for investigations of glucocorticoid homeostasis, which is regulated both at systemic and tissue-specific levels in an interdependent manner.[378] Additionally, in vivo settings allow for influences of diet and physical activity on muscle physiology, which are challenging to replicate in alternative experimental settings. It must be acknowledged that utilisation of animal models to study human disease encompasses certain limitations. Principal limitations are species differences in physiology and difficulty with measuring outcomes that reflect functional performance. Nevertheless, animal studies offer significant advantages over human studies that justify their use. The advantages include faster and cheaper delivery, avoidance of potential harm to humans related to interventions, easier experimental control of time-course and disease severity, straightforward access to deep tissues and simpler ascertainment of multiple outcome measures. Hence, an animal experiment is an effective experimental approach to evaluating the fundamental research hypothesis, which states that overactivity of 11βHSD1 in skeletal muscle contributes to muscle wasting in chronic kidney disease and that inhibition of 11βHSD1 reduces muscle wasting in kidney disease.

Selection of the most appropriate animal model is a critical consideration for in vivo experiments. Multiple rodent models of renal impairment have been characterised in the literature.[220, 379-381] Of these, induction of renal injury through administration of dietary adenine combines a unique set of characteristics that make it suitable for this study. Adenine induces kidney-specific injury and persistent reduction in renal function through deposition of 2,8-dihydroxyadenine crystals in renal tubules.[344] The severity of renal impairment is tuneable through adjustment of exposure dose and exposure duration.[342, 343] The resulting mouse model replicates systemic complications of CKD, including cardiovascular changes, secondary hyperparathyroidism, anaemia and importantly muscle atrophy.[342, 343, 346, 347, 382, 383] Reduction in muscle size and muscle fibre size have previously been validated for the adenine diet model.[345, 384-386] Furthermore, previous studies have demonstrated that the muscle phenotype is amenable to intervention independent of renal dysfunction in this model.[175, 384] Finally, the adenine model is technically simpler to implement compared to alternative models, such as the 5/6-nephrectomy model, with lower variability and lower animal losses.[345] The adenine diet-induced model is therefore chosen as the most suitable model for this study.

Experimental suppression of 11βHSD1 activity in vivo will be achieved through germline deletion of 11βHSD1. Germline deletion provides meaningful advantages compared to alternative pharmacological approaches to suppress 11βHSD1 activity, namely unambiguous specificity for 11βHSD1 type 1 over 11βHSD type 2, and independence from altered handling of drugs in renal impairment. Mice with genetic 11βHSD1-KO have been established previously and have been characterised extensively in the literature.[328, 329, 387] The phenotype of

these genetically altered animals has minimal differences to wild-type animals at baseline conditions. However, 11βHSD1-KO animals exhibit resilience to metabolic stressors and susceptibility to inflammatory insults, highlighting the relevance of 11βHSD1 in disease settings.[263, 328, 329, 387] A similar effect pattern has been reported for skeletal muscle outcomes, indicating differential effects of 11βHSD1 suppression on skeletal muscles depending on biological context.[263, 329, 351] Yet, the effects of 11βHSD1-KO on skeletal muscle phenotype in the context of renal impairment, which combines metabolic stress and inflammatory activation, remain unclear. The adenine diet model has been implemented in 11βHSD1-KO mice in one previous study, which described equivalent renal dysfunction compared to wild-type mice.[259] This suggests that 11βHSD1-KO does not interfere with the adenine diet model of renal impairment. Summing up these considerations, genetic deletion of 11βHSD1 represents a suitable and effective experimental strategy for suppressing 11βHSD1 function in this in vivo study.

3.1.2 Chapter aim and objectives

The following animal experiment will test the therapeutic potential of 11βHSD1 suppression to counter muscle atrophy in renal impairment in vivo. It will also examine the role of 11βHSD1 in the pathophysiology of muscle atrophy in renal impairment in vivo, and explore the ramifications on metabolism, inflammation and glucocorticoid homeostasis when 11βHSD1 function is suppressed in the context of renal impairment.

The overall aim for this research chapter is:

1. Determine the therapeutic potential and accompanying physiological changes of 11βHSD1 suppression to prevent muscle atrophy in a mouse model of persistent renal impairment.

This aim can be broken down into specific sequential objectives:

a) Establish a mouse model of persistent renal impairment with accompanying skeletal muscle atrophy using adenine-supplemented diet
b) Test the efficacy of genetic 11βHSD1 deletion to reduce muscle atrophy in mice with persistent renal impairment
c) Explore the physiological consequences of genetic 11βHSD1 deletion in mice with persistent renal impairment in relation to metabolism, inflammation and glucocorticoid homeostasis

3.2 Results

3.2.1 Model optimisation: 0.15% adenine diet produces a tolerable model of renal impairment and skeletal muscle atrophy in male C57/Bl6 mice

The initial steps of the animal experiment focused on establishing a model of sustained renal impairment that is accompanied by measurable skeletal muscle atrophy and tolerated without undue adverse effects. A model with these characteristics is a prerequisite to investigating the role of 11βHSD1 for muscle atrophy related to renal impairment in vivo and assessing 11βHSD1 blockade as a therapeutic strategy for this indication (research aim 1). Based on existing literature, the experimental approach of supplementing the diet for mice with adenine was selected.[259, 342, 384] This model was tested along the parameters of kidney dysfunction, skeletal muscle atrophy and tolerability.

Male 9-week-old C57/Bl6 mice receiving 0.18% adenine in their diet for 7 weeks develop a clear reduction in renal filtration function, as indicated by a 6.4-fold elevation of serum creatinine compared to animals receiving normal chow (10.4±5.7µmol/L and 66.3±3.2µmol/L for normal chow and 0.18% adenine respectively, $p<0.0001$; Figure 3.1C). Animals receiving 0.18% adenine also develop distinct features of skeletal muscle atrophy with 20.1% reduced gross quadriceps muscle weight (256.5±28.4mg and 205.0±9.0mg for normal chow and 0.18% adenine respectively, $p=0.011$; Figure 3.1D). However, adenine diet supplementation at this dosage and duration leads to notable loss of body weight. Employing a body weight loss of ≥20% relative to baseline as a humane endpoint to limit welfare burden, the experimental protocol could not be completed for 9 of 12 animals (Figure 3.1A). Weight loss was most

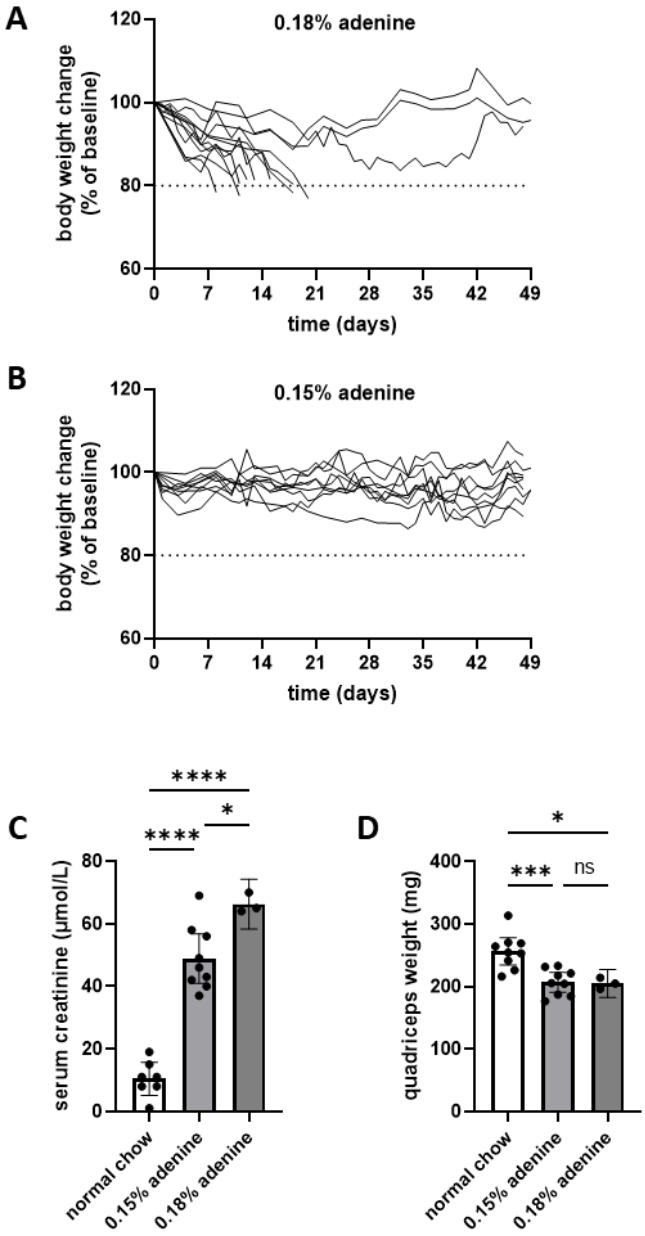


Figure 3.1 Dose titration of adenine in diet to generate a mouse model with renal impairment and muscle atrophy.

A) *Body weight relative to baseline for individual mice receiving 0.18% adenine in diet for up to 7 weeks. Animals were culled and data censored when weight loss reached ≥20% as per pre-specified welfare severity limits.* ***B)*** *Relative change in body weight of mice receiving 0.15% adenine in diet over 7 weeks.* ***C)*** *Serum creatinine concentration of animals after 7 weeks of normal chow, 0.15% dietary adenine and 0.18% dietary adenine.* ***D)*** *Quadriceps muscle weight of animals after 7 weeks of normal chow, 0.15% dietary adenine and 0.18% dietary adenine. All data are from male wild-type C57Bl/6 mice aged 8-9 weeks at baseline. Analysis by one-way ANOVA with Tukey's multiple comparisons test; ns p>0.05, * p<0.05, *** p<0.001, **** p<0.0001.*

pronounced within the first 3 weeks of adenine diet administration. No other adverse events were observed besides loss of body weight. Administration of 0.18% adenine diet over 7 weeks therefore produces a model with suitable renal and skeletal muscle phenotype, but the extent of accompanying weight loss limits its suitability as an experimental model.

The dosage of adenine in the diet was reduced from 0.18% to 0.15% to refine the experimental protocol and improve tolerability. Administration of 0.15% adenine in the diet over 7 weeks remains effective at reducing kidney function and skeletal muscle mass in male B57/Bl6 mice. Serum creatinine rises to 4.7-fold levels compared to control animals (10.4±5.7µmol/L and 48.9±10.3µmol/L for normal chow and 0.15% adenine respectively, $p<0.0001$; Figure 3.1C). Quadriceps muscle weight declines by 19.4% compared to control animals (256.5±28.4mg and 206.8±20.7mg for normal chow and 0.15% adenine respectively, $p<0.001$; Figure 3.1D). Contrary to 0.18% adenine, administration of 0.15% adenine is well tolerated. No experimental animals reached the humane endpoint of ≥20% body weight loss relative to baseline within the duration of the experiment (Figure 3.1B). No other adverse events were observed. These results validate the experimental protocol of 0.15% adenine administration via the diet over 7 weeks to produce a suitable model of renal impairment with muscle atrophy and acceptable tolerability. It is therefore an appropriate model to investigate the role of 11βHSD1 and its potential as a therapeutic target for muscle atrophy in persistent renal impairment. All subsequent animal experiments of renal impairment are based on 0.15% adenine diet administration over 7 weeks, unless explicitly stated otherwise.

3.2.2 Kidney phenotype: wild-type and 11βHSD1 mice develop comparable levels of renal impairment

An animal experiment using the adenine-diet model was conducted to investigate the contribution of steroid activation by 11βHSD1 towards muscle atrophy in renal impairment and test its inhibition as a therapeutic strategy. The experiment used male 9-week-old C57/Bl6 mice and consisted of four experimental groups (n=9-11 per group). Wild-type animals or animals with genetic deletion of 11βHSD1 (11βHSD1-KO) received diet with 0.15% adenine for 7 weeks to give two groups with renal impairment. Age-matched wild-type or 11βHSD1-KO animals receiving normal chow over the same period provided two control groups.

The renal phenotype was characterised in greater detail and compared between wild-type and 11βHSD1-KO animals. This was done to provide a foundation for interpreting any subsequent experimental results and exclude divergent effects of adenine on wild-type and 11βHSD1-KO animals. Average body weights decrease slightly with the adenine diet in both wild-type and 11βHSD1-KO groups (relative weight change from baseline -3.6±4.5% and -7.9±5.2% for WT-adenine and 11βKO-adenine groups respectively; Figure 3.2A). Average body weights in the control groups rise gradually over the experimental period (relative weight change from baseline +20.5±9.0% and +18.4±4.1% for WT-control and 11βKO-control groups respectively; Figure 3.2A). The final body weights are accordingly significantly higher in control groups than in the adenine-treated groups, without significant differences between wild-type and 11βHSD1-KO animals (Figure 3.2B).

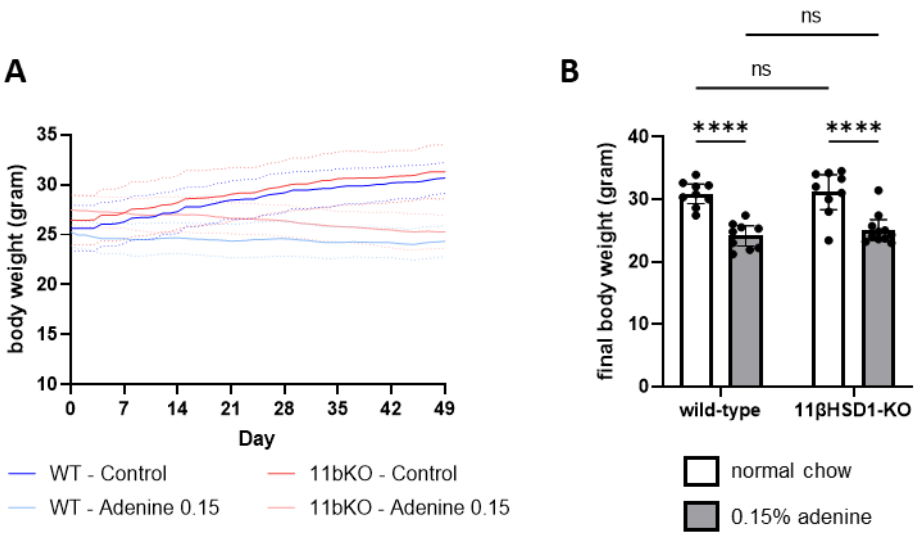


Figure 3.2 Body weight for wild-type and 11βHSD1-KO mice receiving normal chow or 0.15% adenine diet over 7 weeks.

A) *Average body weights during the experimental period. Solid line indicates 7-day running average for each experimental group, dotted line indicates 95% confidence interval. (n=11 for 11βKO – adenine group, n=9 for other groups)* ***B)*** *Final body weights at the end of the experimental protocol. Analysis by two-way ANOVA with Tukey's multiple comparisons test; ns p>0.05, **** p<0.0001.*

Adenine diet treatment induces clear macroscopic changes in mouse kidneys with a shrunken and irregular surface appearance (Figure 3.3A). Renal atrophy is confirmed by measurements of organ weights, with adenine leading to -45.6% relative kidney weight reduction in wild-type mice and -22.5% in 11βHSD1-KO mice (Figure 3.3B). The results show that kidney weights are slightly lower in wild-type than in 11βHSD1-KO animals following adenine treatment, which was statistically significant (diet effect p<0.0001, genotype effect p=0.032 & interaction p=0.026 in 2-way ANOVA; Figure 3.3B). Significant elevation of serum creatinine (fold-change 4.7x and 4.1x for WT and 11βKO animals respectively, p<0.0001 for both changes; Figure 3.3C) and serum urea (fold-change 3.2x and 2.8x for WT and 11βKO animals respectively, p<0.0001 for both changes; Figure 3.3D) confirm solute retention and reduced kidney filtration function

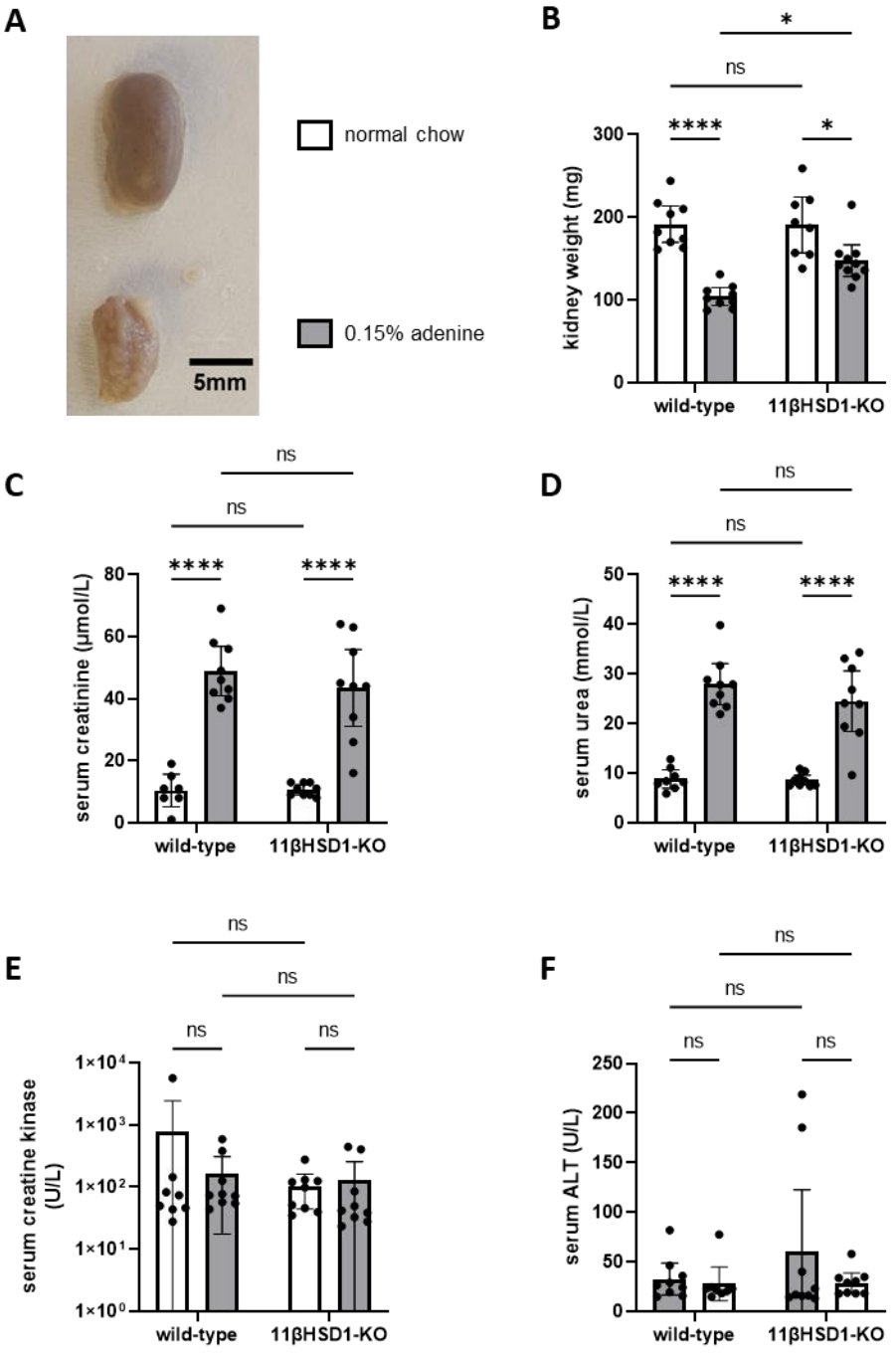


Figure 3.3 Renal phenotype of wild-type and 11βHSD1-KO mice after 7 weeks of normal chow or 0.15% adenine diet.

A) *Macroscopic appearance of kidneys from wild-type animals receiving normal chow (top) and 0.15% dietary adenine (bottom).* ***B)*** *Kidney weights,* ***C)*** *serum creatinine concentration,* ***D)*** *serum urea concentration,* ***E)*** *serum creatine kinase concentration, and* ***F)*** *serum alanine transferase concentration of experimental animals. Analysis by two-way ANOVA with Tukey's multiple comparisons test; ns p>0.05, * p<0.05, **** p<0.0001.*

in adenine-treated groups compared to control groups. There is no significant difference based on genotype for serum creatinine changes (diet effect $p<0.0001$, genotype effect $p>0.05$ & interaction $p>0.05$ in 2-way ANOVA; Figure 3.3C) or serum urea changes (diet effect $p<0.0001$, genotype effect $p>0.05$ & interaction $p>0.05$ in 2-way ANOVA; Figure 3.3D). Serum creatine kinase and serum alanine transferase were measured as respective indicators of direct skeletal muscle or liver tissue damage. Neither marker changes significantly with adenine treatment, indicating no direct toxic effects of adenine on these tissues (Figure 3.3E&F).

Renal histology was examined as an additional assessment of adenine-mediated effects (Figure 3.4). Kidneys from adenine-treated animals show replacement of normal tubular structures, that comprise cells staining with eosin in their cytoplasm, with more densely packed cells, that contain little cytoplasm and stain prominently with haematoxylin. This appearance is consistent with inflammatory infiltrates and fibrosis. Adenine-exposed kidneys furthermore show fewer intact glomeruli and contain larger vacuoles, suggestive of dilated tubules. Finally, individual crystals are apparent in adenine-exposed kidneys. No substantial difference in the histological appearance of adenine-exposed kidneys from wild-type or 11βHSD1-KO animals is detectable on H&E staining.

The characterisation of renal phenotype by macroscopic, biochemical and histological markers therefore confirms renal atrophy, impaired renal function and disruption of normal kidney architecture with adenine exposure. Apart from an isolated difference in kidney weights, renal changes with adenine exposure are comparable in wild-type and 11βHSD1-KO animals.

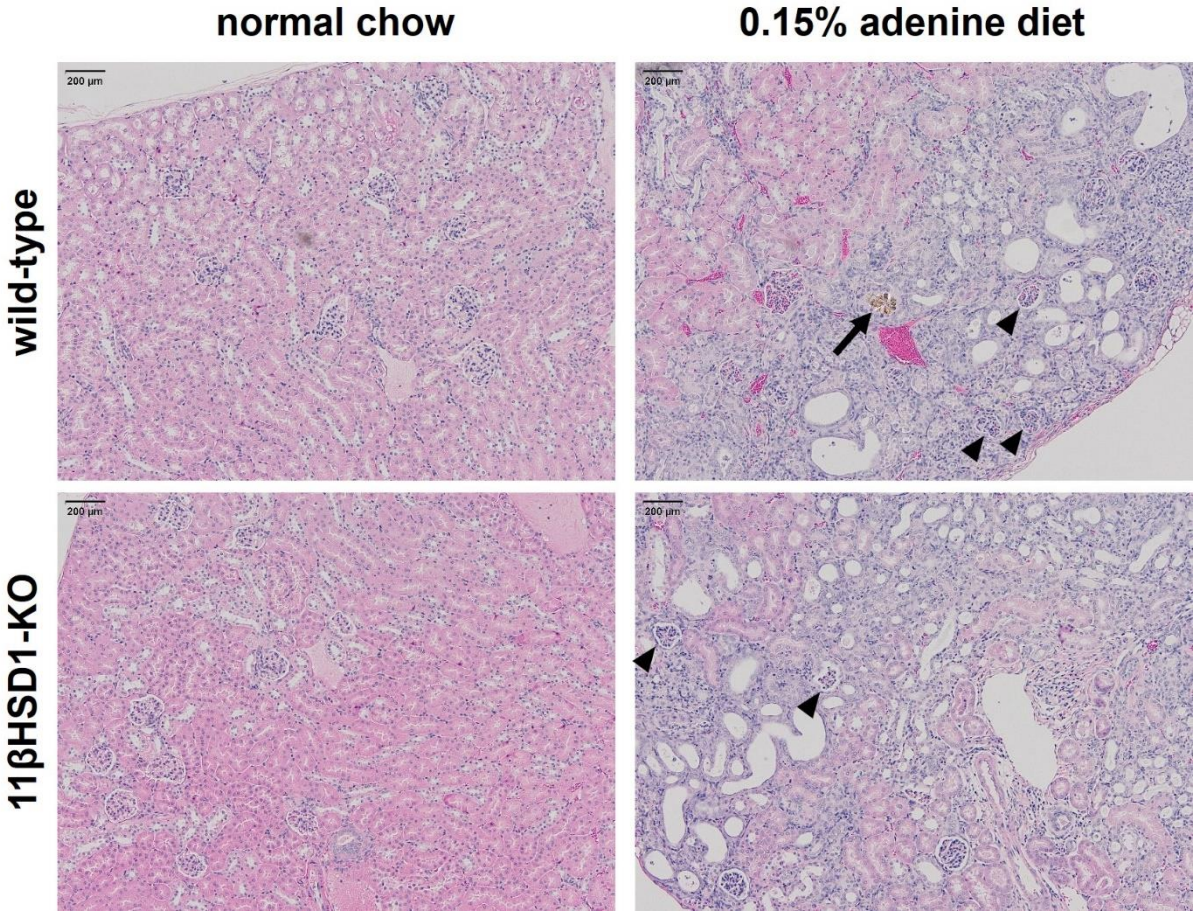


Figure 3.4 Renal histology of wild-type and 11βHSD1-KO mice after 7 weeks of normal chow or 0.15% adenine diet.

Arrowheads indicate examples of fibrosed glomeruli, arrow indicates examples of crystal deposition. Haematoxylin & eosin staining, 100x magnification.

3.2.3 Muscle phenotype: genetic deletion of 11βHSD1 does not prevent muscle atrophy in the adenine-diet model of renal impairment

The skeletal muscle phenotype constitutes the outcome of principal interest in this animal experiment. The adenine-diet model has been validated to entail a phenotype of skeletal muscle atrophy. Examination of the skeletal muscle phenotype in wild-type and 11βHSD1-KO will provide insights into the role of 11βHSD1 for skeletal muscle changes in kidney disease and its potential as a therapeutic target. A range of complementary methodologies will

construct a comprehensive picture of the skeletal muscle phenotype, including measurements of hindlimb muscle weights, muscle fibre size, histological architecture, in vivo grip strength, and mRNA expression of anabolic and catabolic regulators.

The first assessment of skeletal muscle phenotype in adenine-treated mice is based on the weight of several dissected hindlimb muscles, namely quadriceps, soleus and tibialis anterior. Quadriceps muscle weight of wild-type animals is 19.4% lower in adenine-fed mice compared to control mice (256.5±28.4mg and 206.8±20.7mg for WT-control and WT-adenine respectively, $p<0.001$, Figure 3.5A). For 11βHSD1-KO animals, the change in quadriceps muscle weight is similar with a 16.0% reduction in adenine-fed compared to control mice (234.7±37.9mg and 197.2±18.5mg for 11βKO-control and 11βKO-adenine respectively, $p<0.001$, Figure 3.5A). No significant difference exists in quadriceps muscle weights from adenine-treated wild-type or 11βHSD1-KO mice ($p>0.05$ in Tukey's multiple comparisons test or in sensitivity analysis using independent t-test without adjustment for multiple comparisons). Measurements of soleus muscle weight replicate the pattern of quadriceps muscle weight changes. Adenine diet reduces soleus weight in wild-type and 11βHSD1-KO animals to a similar extent, without differences between genotypes (relative change -20.2% and -19.0% for WT and 11βKO groups respectively, $p<0.01$ for comparisons by diet and $p>0.05$ for comparisons by genotype; Figure 3.5B). Tibialis anterior muscle appears protected from muscle atrophy in the adenine diet model, without significant reductions in either genotype (relative change -3.3% and -5.7% for WT and 11βKO groups respectively; Figure 3.5C).

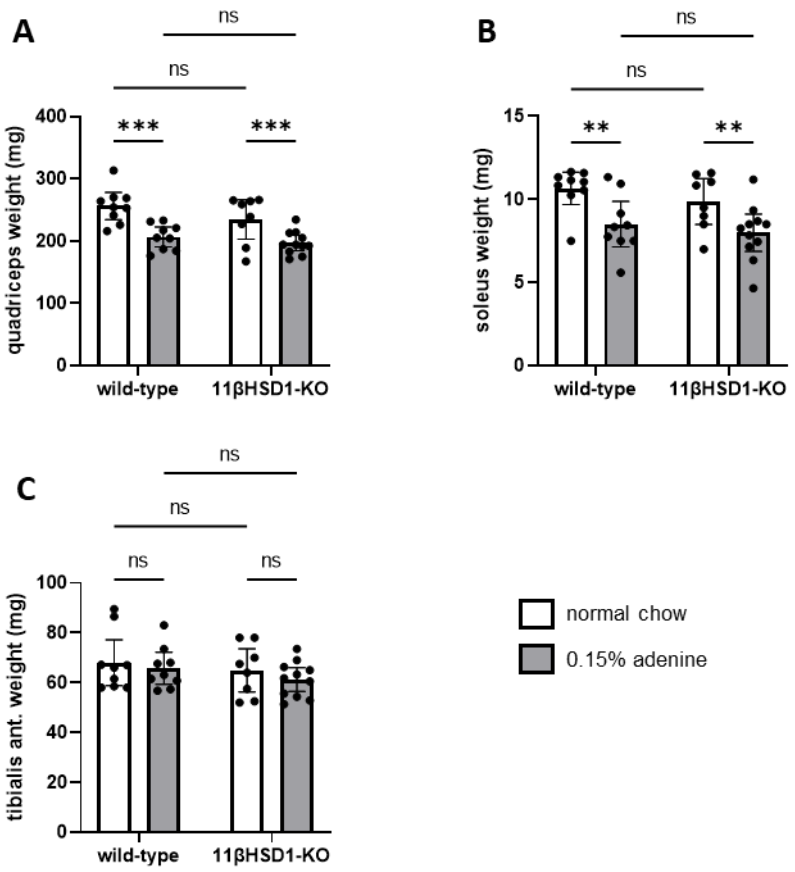


Figure 3.5 ***Skeletal muscle weights of wild-type and 11βHSD1-KO mice after 7 weeks of normal chow or 0.15% adenine diet.***

A) *Quadriceps muscle weights,* ***B)*** *soleus muscle weights, and* ***C)*** *tibialis anterior muscle weights of experimental animals. Analysis by two-way ANOVA with Tukey's multiple comparisons test; ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

The second assessment of skeletal muscle phenotype examines myofibre size in quadriceps muscle from representative histological images of quadriceps muscle in cross-sectional profile stained with haematoxylin & eosin (Figure 3.6). Myofibre size distribution is essentially congruent in wild-type and 11βHSD1-KO animals in the control condition, excluding a baseline phenotype difference (Figure 3.7A). Adenine diet induces a left shift in myofibre size distribution in both wild-type and 11βHSD1-KO mice, indicating myofibre atrophy in both genotypes (Figure 3.7B&C). When myofibre size distribution of adenine-treated wild-type and

adenine-treated 11βHSD1-KO animals are compared, there is no detectable difference (Figure 3.7D). The alternative marker of mean myofibre size corresponds with the pattern of changes observed for myofibre size distribution, reduced in adenine-fed groups and unchanged by 11βHSD1 knock-out (diet effect $p<0.001$, genotype effect $p>0.05$ & interaction $p>0.05$ in 2-way ANOVA; Figure 3.7E). Besides myofibre atrophy described above, no changes in muscle architecture are evident. As far as can be determined in H&E-stained sections, there is no evidence of infiltration of other cell types, significant lipid deposition or fibrosis (Figure 3.6).

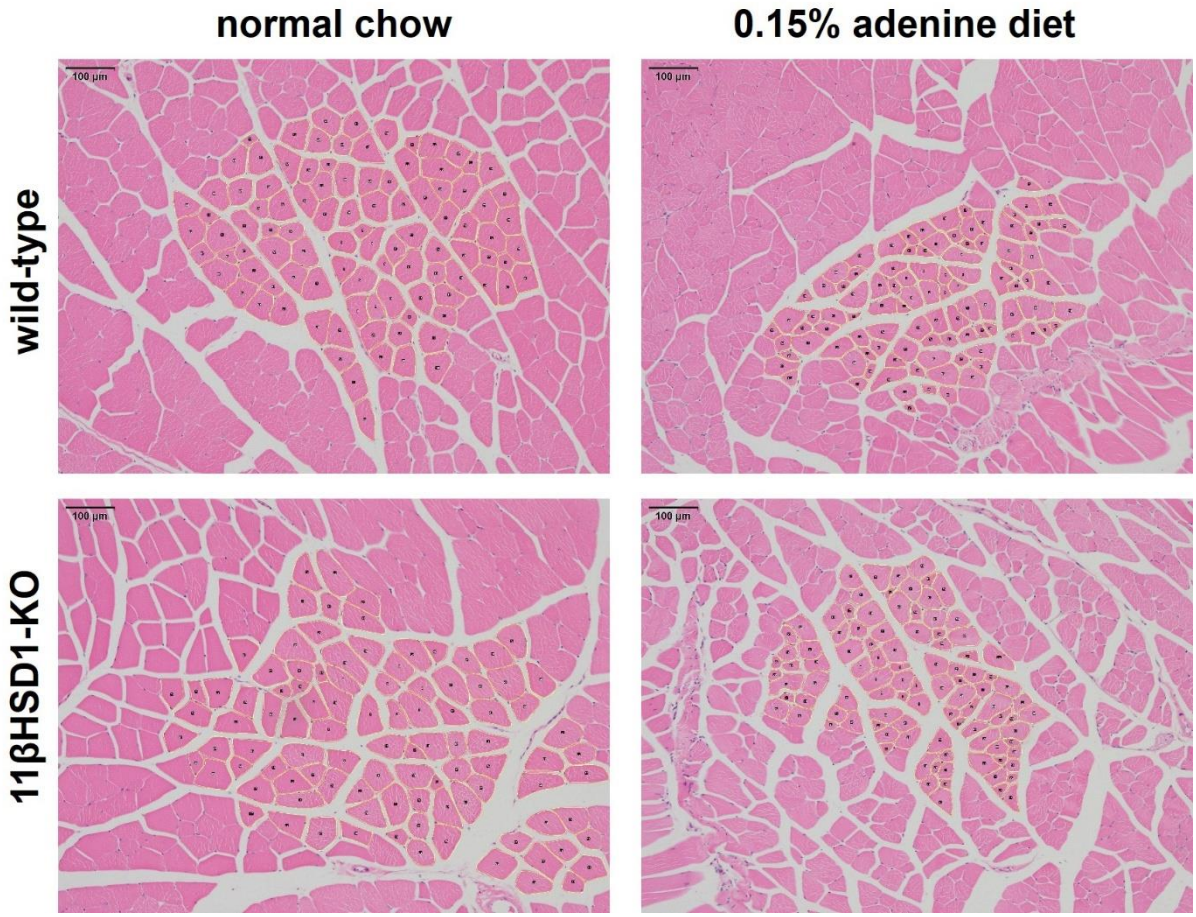


Figure 3.6 Quadriceps muscle histology of wild-type and 11βHSD1-KO mice after 7 weeks of normal chow or 0.15% adenine diet.

Representative images of cross-sectional sections through the mid-belly of quadriceps muscles. Mark-up represents basis for fibre size measurements. Haematoxylin & eosin staining, 100x magnification.

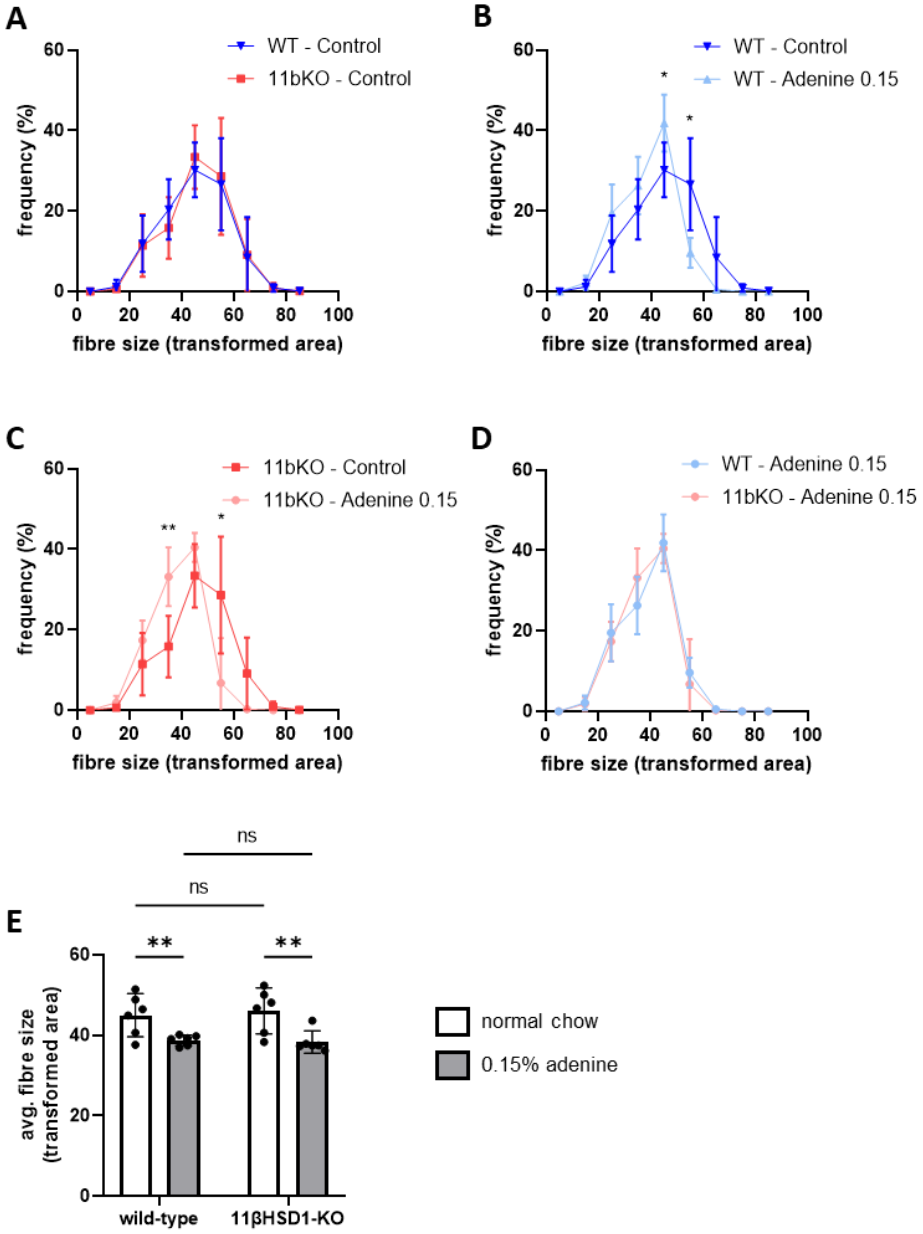


Figure 3.7 Quadriceps muscle fibre size of wild-type and 11ϐHSD1-KO mice after 7 weeks of normal chow or 0.15% adenine diet.

*Comparison of muscle fibre size distributions for **A)** WT/control vs 11ϐHSD1-KO/control, **B)** WT/control vs WT/adenine, **C)** 11ϐHSD1-KO/control vs 11ϐHSD1-KO/adenine and **D)** WT/adenine vs 11ϐHSD1-KO/adenine. Cross-sectional area of individual fibres was determined on H&E-stained sections using ImageJ software and transformed with the square root function for analysis. Summary data for 6 animals per group is shown, based on 300 fibre size measurements across three different regions for each animal. Individual data points are compared by 2-tailed t-test. **E)** Average quadriceps muscle fibre size of experimental animals. n=6 per group, data obtained as described above. Analysis by two-way ANOVA with Tukey's multiple comparisons test; ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

Combined hind and foreleg grip strengths were assessed as a third aspect of skeletal muscle phenotype. As an in vivo marker of muscle function, it was measured at multiple timepoints to track changes over the time of the experimental protocol. Grip strength measurements do not identify any significant differences between experimental groups, neither by diet nor genotype, at baseline or any subsequent timepoints (Figure 3.8A). Grip strength at the end of the experimental protocol is not significantly different among the four experimental groups (WT-control: 162±16gram; WT-adenine: 175±38gram; 11βKO-control: 144±19gram; 11βKO-adenine: 156±27gram; p>0.05 for all comparisons in Tukey's multiple comparisons test; Figure 3.8B).

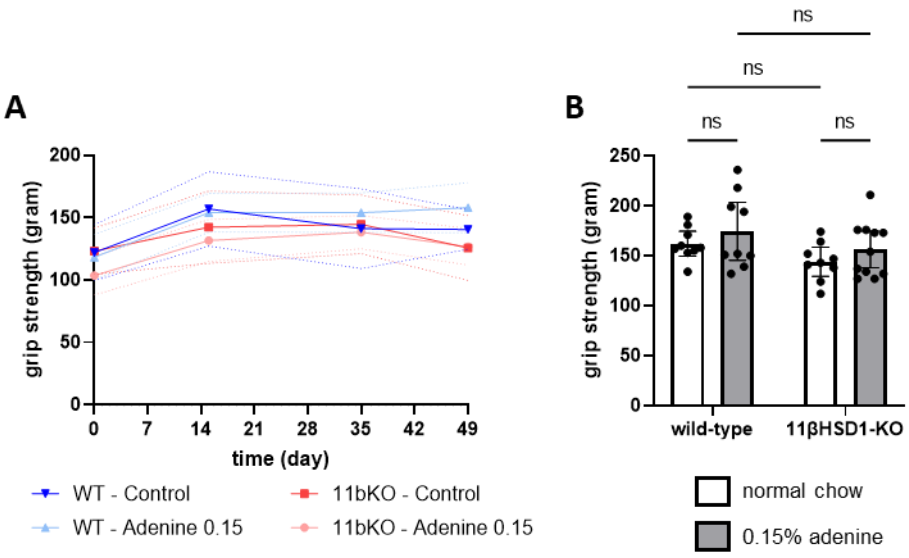


Figure 3.8 Grip strength measurements of wild-type and 11βHSD1-KO mice receiving normal chow or 0.15% adenine diet for 7 weeks.

A) *Longitudinal trend in grip strength measured at baseline, day 15, day 35 and end of the 7-week protocol. Data points show group means for maximal grip strength of mice and dotted lines indicate the 95% confidence interval. Only animals with complete datasets are included in this analysis (n=4 for wt – control, n=4 for 11βKO – control, n=6 for WT – adenine, n=8 for 11βKO – adenine).* ***B)*** *Maximal grip strength of animals at the end of the experimental protocol. Analysis by two-way ANOVA with Tukey's multiple comparisons test; ns p>0.05.*

Finally, muscle phenotype was assessed by determining mRNA expression of anabolic and catabolic genes in quadriceps muscle. There is a small but statistically significant reduction in mRNA expression of the catabolic gene Fbxo32 after 7 weeks on adenine diet (1.3x negative fold-change, p=0.041; Figure 3.9A). Downregulation of Fbxo32 is similar in wild-type and 11βHSD1-KO animals (1.6x negative fold-change, p=0.041; Figure 3.9A). No significant differences in mRNA levels of the catabolic genes Foxo1 or Trim63, anti-anabolic myostatin or anabolic Igf1 occur in any experimental group (Figure 3.9B-E).

Evaluation of measures of muscle size, architecture, function and metabolic regulation confirms a phenotype of reduced skeletal muscle mass following exposure to adenine diet. Adenine at 0.15% over 7 weeks leads to atrophy in quadriceps and soleus muscles, with relative sparing of tibialis anterior muscle. Myofibre size reduction in quadriceps corroborates observed loss of gross muscle weight. With the implemented experimental protocol, no detectable changes in grip strength are observed, neither are catabolic mRNA markers upregulated at the experimental endpoint. Importantly, genetic deletion of 11βHSD1 does not alter the skeletal muscle phenotype in the adenine-diet induced model of renal impairment.

3.2.4 Metabolic phenotype: genetic deletion of 11βHSD1 improves glucose tolerance independent of renal impairment

Impaired insulin signalling and metabolic imbalance are features of renal impairment that contribute to muscle atrophy and dysfunction.[95, 141] Previous studies indicated an improvement in these markers with genetic deletion of 11βHSD1 [259, 328], providing a rationale to probe the metabolic phenotype of experimental animals in further detail.

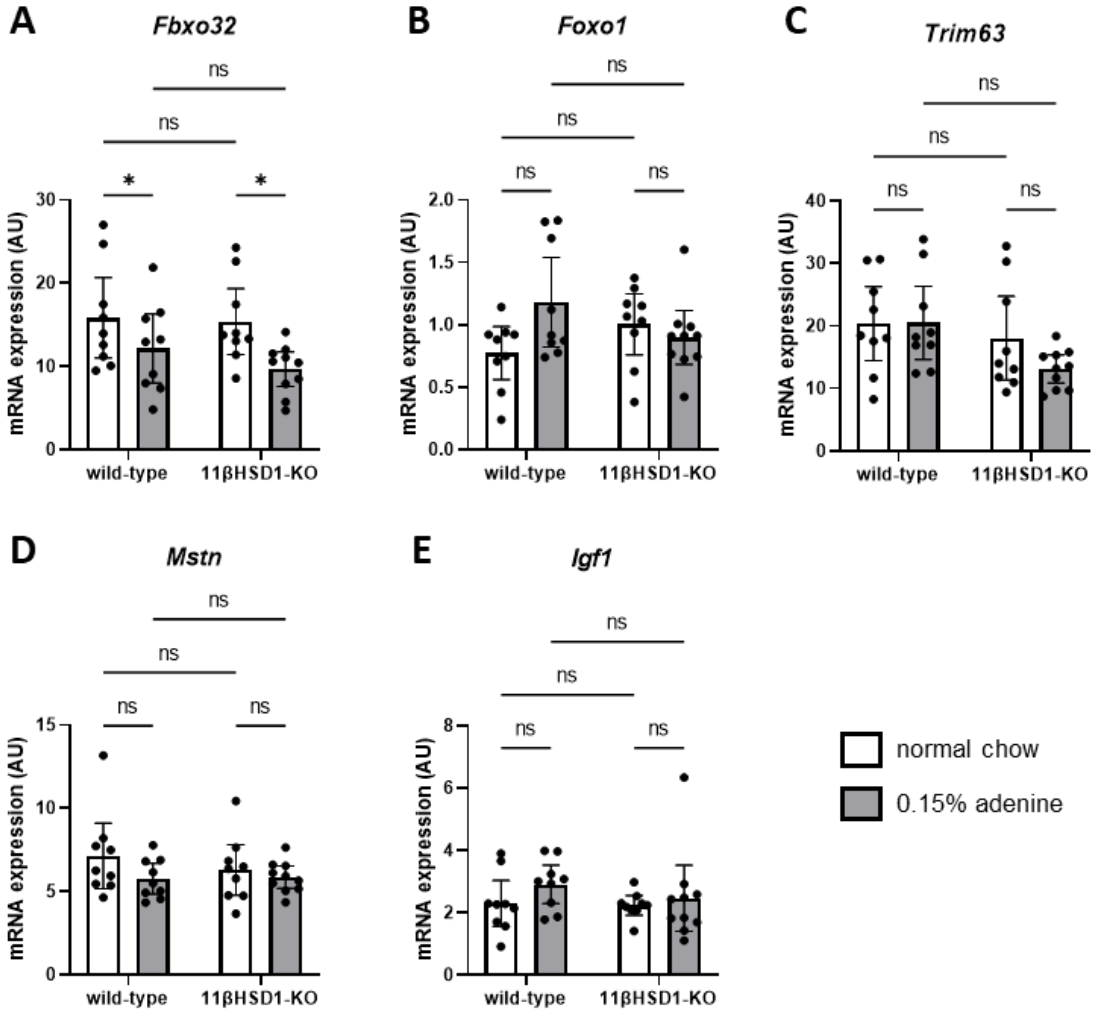


Figure 3.9 mRNA expression of anabolic and catabolic genes in quadriceps muscles of wild-type and 11βHSD1-KO mice after 7 weeks of normal chow or 0.15% adenine diet. ***A-C)*** *Expression levels of catabolic genes Fbxo32, Foxo1 & Trim63,* ***D)*** *anti-anabolic gene Mstn, and* ***E)*** *anabolic gene Igf1 in mouse quadriceps muscle. mRNA expression level was determined by rtPCR and normalised to the housekeeper gene Gapdh. Analysis by two-way ANOVA with Tukey's multiple comparisons test; ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

Fasting blood glucose levels are significantly lower in the adenine-fed groups (mean difference for WT groups: -3.6mmol/L, p<0.001; mean difference for 11βKO groups: -2.4mmol/L, p=0.018; Figure 3.10A), without significant differences between wild-type and 11βHSD1-KO animals (mean difference in control condition +0.9mmol/L; mean difference in adenine

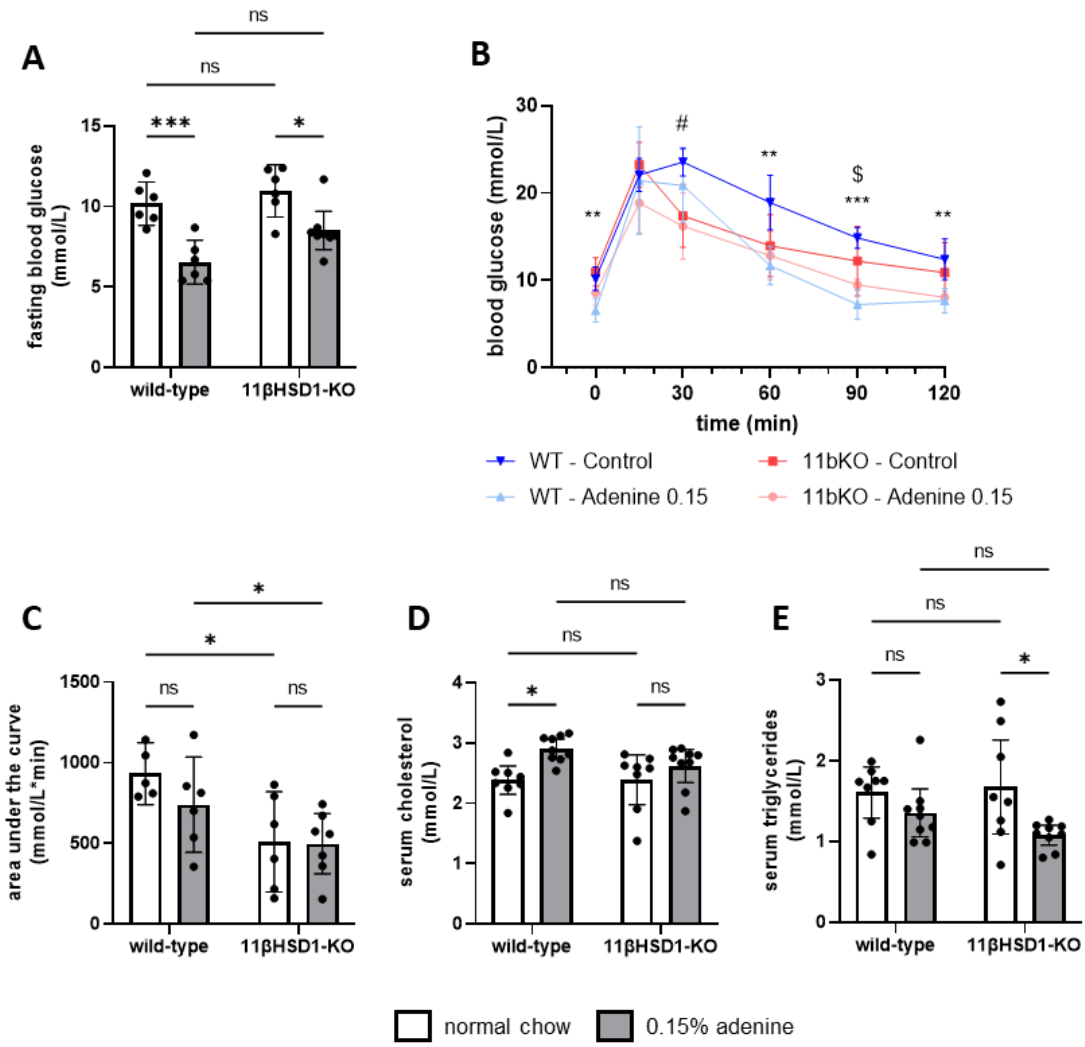


Figure 3.10 Glucose and lipid phenotype of wild-type and 11βHSD1-KO mice receiving normal chow or 0.15% adenine diet.

A) *Blood glucose concentration after fasting for 5 hours at week 5 in the experimental protocol.* ***B)*** *Glucose tolerance test data: change in blood glucose following intraperitoneal injection of 2mg/kg glucose to fasted animals at week 5 in the experimental protocol. Data points show group means for the respective timepoint and error bars indicate the 95% confidence interval. (n=7 for 11βKO – adenine, n=6 for other groups). Pairwise comparison of means using multiple unpaired t-tests with correction for multiple comparisons using the Holm-Sidak method. ** p<0.01 & *** p<0.001 for WT-control vs. WT-adenine; # p<0.05 for WT-control vs. 11βKO-control; $ p<0.05 for WT-adenine vs KO-adenine.* ***C)*** *Areas under the curve for glucose tolerance test data. Areas under the curve were calculated in GraphPad Prism for individual animals using fasting glucose as the baseline.* ***D)*** *Serum total cholesterol concentration and* ***E)*** *serum triglyceride concentration at the end of the experimental protocol. Unless otherwise specified, analysis by two-way ANOVA with Tukey's multiple comparisons test; ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001.*

condition +2.0mmol/L, p>0.05 for both; Figure 3.10A). Upon an intraperitoneal 2mg/g glucose challenge, blood glucose returns to lower levels more quickly in wild-type animals on adenine diet compared to normal chow (Figure 3.10B). Area under the curve after, adjusted for differing baseline glucose, is also marginally lower in adenine-fed than chow-fed wild-type animals, but the difference is not statistically significant (relative difference -20.7%, p>0.05; Figure 3.10C). Among 11βHSD1-KO animals, glucose tolerance test parameters are similar for adenine-fed and chow-fed groups (relative difference -2.3%, p>0.05; Figure 3.10C). Notably, 11βHSD1-KO animals exhibit an improved glucose tolerance profile compared to wild-type animals. Both in the chow-fed condition and in the adenine-fed condition, area under the curve is lower in 11βHSD1-KO mice than in wild-type mice (relative difference in control condition -45.4%, p=0.017; relative difference in adenine condition -33.7%, p=0.017; Figure 3.10C).

Finally, measurement of serum lipids provides mixed results. Serum cholesterol or triglycerides are not significantly different between wild-type and 11βHSD1-KO animals in either adenine or control condition (Figure 3.10D&E). Serum cholesterol increases significantly with the adenine condition in wild-type animals (mean difference +0.52mmol/L, p=0.021, Figure 3.10D), but not in 11βHSD1-KO animals (mean difference +0.23mmol/L, p>0.05, Figure 3.10D). Conversely, serum triglycerides decrease significantly with the adenine condition in 11βHSD1-KO animals (mean difference -0.60mmol/L, p=0.041, Figure 3.10E), but not in wild-type animals (mean difference -0.25mmol/L, p>0.05, Figure 3.10E).

Summarising the metabolic phenotype results, the most notable finding is an improvement in glucose tolerance in 11βHSD1-KO mice, regardless of adenine diet exposure. Furthermore,

animals with adenine-induced renal impairment have lower fasting glucose, independent of 11βHSD1 genotype. Serum cholesterol may be increased and serum triglyceride levels may be decreased in this adenine-diet model.

3.2.5 Inflammation phenotype: renal inflammation is similar in wild-type and 11βHSD1 mice without evidence of muscle inflammation

Inflammation is another important aspect of renal impairment that influences metabolism and muscle physiology.[55, 138, 139] Given also the reciprocal interactions between glucocorticoids and inflammation [179, 310], an investigation of inflammatory changes in this animal study is highly pertinent. Tissue-specific examination of inflammatory markers showed significant pro-inflammatory changes in kidneys from adenine-fed animals. Expression of mRNA for proinflammatory cytokine genes TNFα, Il1β and Il6 is greatly upregulated in kidneys from both wild-type and 11βHSD1-KO mice receiving adenine diet (fold-change for WT and 11βKO groups respectively: TNFα 19.2x and 16.9x, $p<0.0001$ for both changes; Il1β 8.1 and 8.3x, $p<0.0001$ for both changes; Il6 38.3x and 15.9x, $p<0.01$ for both changes; Figure 3.11A-C). 11βHSD1 genotype has no statistically significant effect on upregulation of inflammatory markers in kidney tissue (diet effect $p<0.0001$, genotype effect $p>0.05$ & interaction $p>0.05$ in 2-way ANOVA for all three genes). To examine whether localised inflammation in renal tissue has systemic consequences, serum IL6 was measured by ELISA. However, no quantifiable results are available, as Il6 concentrations in serum fell below the assay detection limit (<15pg/ml) in all experimental groups. To look for features of inflammation locally within skeletal muscle tissue, rtPCR for inflammatory cytokine genes was performed on quadriceps mRNA samples. Expression of TNFα and Il6 mRNA is similar in all experimental groups,

regardless of diet condition or genotype (Figure 3.11D&E). Taken together, clear evidence indicates inflammation locally within renal tissue with the adenine diet model, but important acute inflammation markers remain unchanged in skeletal muscle tissue. This pattern of inflammation is not affected by genetic deletion of 11βHSD1.

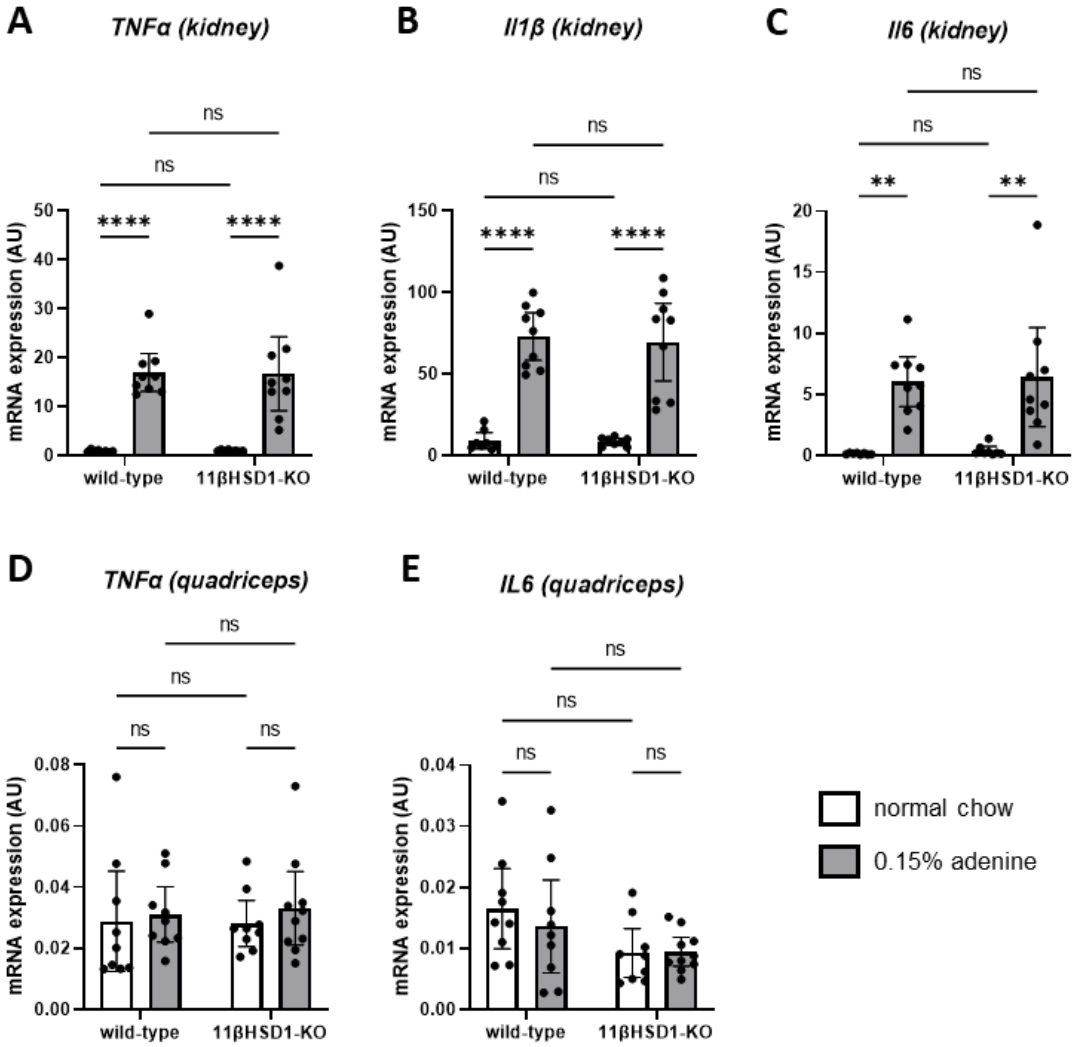


Figure 3.11 mRNA expression of inflammatory cytokine genes in kidney and quadriceps muscles of wild-type and 11βHSD1-KO mice after 7 weeks of normal chow or 0.15% adenine diet.

A-C) *Expression levels for inflammatory cytokines genes TNFα, IL1β and Il6 in kidney tissue, and* ***D-E)*** *for TNFα and Il6 in quadriceps muscle tissue. mRNA expression level was determined by rtPCR and normalised to the housekeeper genes 18S (kidney tissue) or Gapdh (muscle tissue). Analysis by two-way ANOVA with Tukey's multiple comparisons test; ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

3.2.6 Glucocorticoid metabolism: peripheral glucocorticoid metabolism appears unchanged in the model of renal impairment; circulating corticosterone is higher in 11βHSD1-KO mice

A thorough investigation of glucocorticoid metabolism was conducted as the final step in the animal study. This was done to scrutinise the impact of adenine-induced renal impairment on glucocorticoid metabolism and signalling. Furthermore, a clear description of the glucocorticoid phenotype will be necessary for interpreting the role of 11βHSD1 -mediated glucocorticoid metabolism for skeletal muscle atrophy in this experimental model.

Corticosterone, the main active glucocorticoid in rodents, was measured in serum to establish a context for glucocorticoid signalling at the systemic level. Notably, circulating corticosterone is significantly elevated in mice with genetic deletion of 11βHSD1 compared to wild-type animals (mean corticosterone 14.8±11.7ng/mL and 55.0±33.5ng/mL for WT-control and 11βKO-control groups respectively, $p=0.013$; mean corticosterone 20.0±9.6ng/mL and 58.8±32.0ng/mL for WT-adenine and 11βKO-adenine groups respectively, $p=0.017$; Figure 3.12A). In contrast, adenine administration did not affect circulating corticosterone levels (diet effect $p>0.05$, genotype effect $p<0.0001$ & interaction $p>0.05$ in 2-way ANOVA).

To gain deeper insight into glucocorticoid signalling locally within skeletal muscle, a range of muscle-specific assays were performed. Interestingly, expression of 11βHSD1 mRNA is upregulated in quadriceps tissue from adenine-fed wild-type animals compared to control animals (1.7-fold change, $p=0.032$, Figure 3.12B). No expression of 11βHSD1 mRNA is detected in quadriceps muscle from 11βHSD1-KO animals as expected (Figure 3.12B). To explore

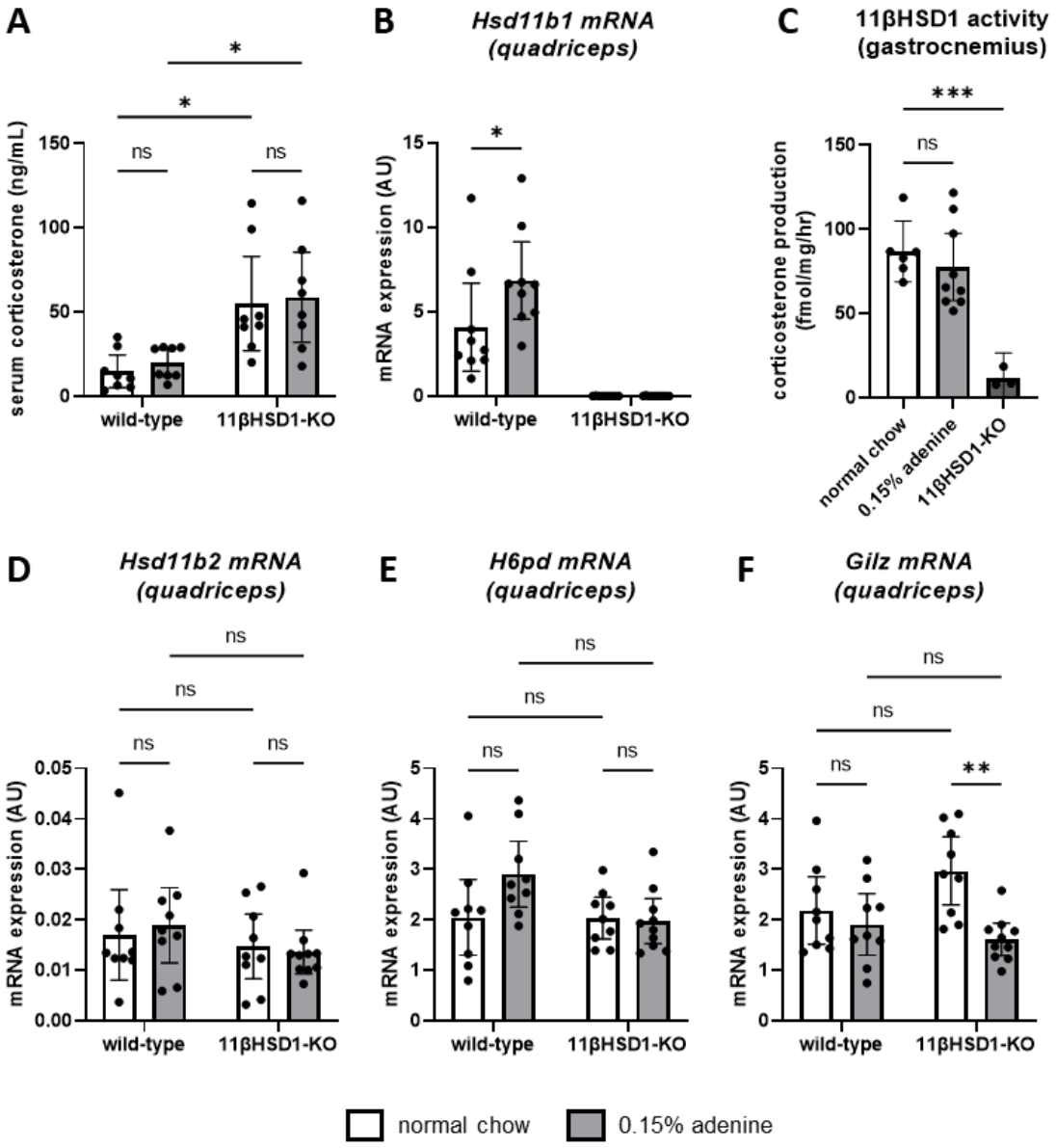


Figure 3.12 Glucocorticoid phenotype in serum and skeletal muscle of wild-type and 11βHSD1-KO mice after 7 weeks of normal chow or 0.15% adenine diet.

A) *Corticosterone concentration in serum (n=8 per group).* ***B)*** *mRNA expression of Hsd11b1 in quadriceps muscle tissue.* ***C)*** *11βHSD1 enzymatic activity in gastrocnemius muscle tissue ex vivo. Data from wild-type animals receiving normal chow (n=6), wild-type animals receiving 0.15% adenine diet (n=9) and from 11βHSD1-KO animals (n=3) respectively. Analysis by one-way ANOVA with Dunnett's post-hoc test.* ***D-F)*** *mRNA expression of Hsd11b2, H6pd and Gilz in quadriceps muscle tissue. mRNA expression level was determined by rtPCR and normalised to the housekeeper gene Gapdh. Unless otherwise specified, analysis by two-way ANOVA with Tukey's multiple comparisons test; ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001.*

whether elevated expression correlated with elevated 11βHSD1 enzymatic activity, conversion of DHC to corticosterone was determined in gastrocnemius muscle samples ex vivo. Contrary to mRNA expression findings, corticosterone production rate appears similar in ex vivo skeletal muscle tissue from wild-type animals with or without renal impairment (86.8±17.1fmol/mg/h for WT-control group and 77.7±26.0fmol/mg/h for WT-adenine group, $p>0.05$, Figure 3.12C). The ex vivo 11βHSD1 activity assay confirms that skeletal muscle tissue from 11βHSD1-KO animals has substantially reduced capacity to convert DHC to corticosterone (corticosterone production 11.6±6.0fmol/mg/h).

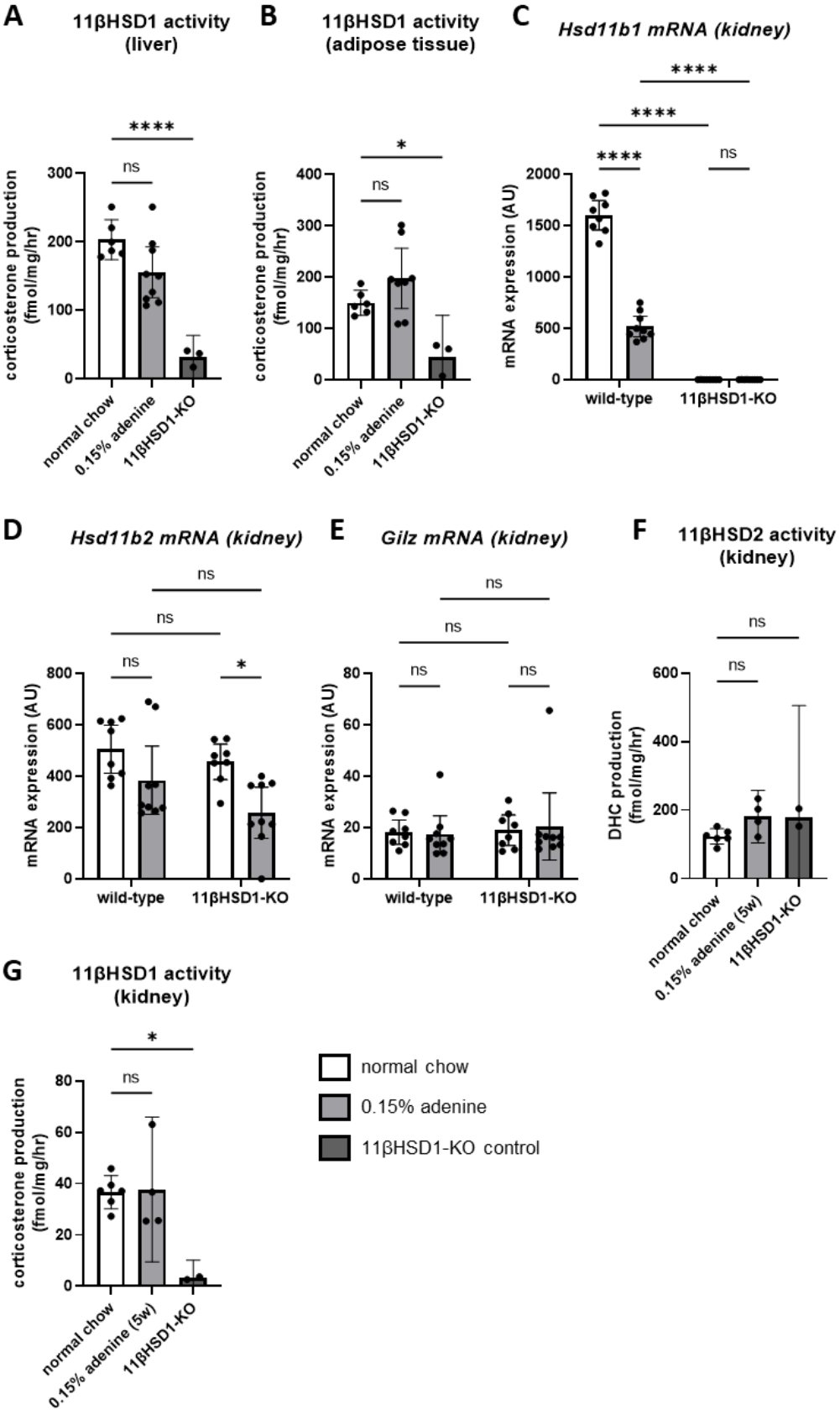
Skeletal muscle mRNA expression of 11βHSD2, which catalyses the reverse reaction of 11βHSD1 , and H6pd, which provides the NADPH co-factor for 11βHSD1 enzymatic activity, remains unchanged among all four experimental groups (Figure 3.12D&E). Finally, the mRNA expression level of Gilz was determined as a surrogate marker of glucocorticoid signal transduction. Gilz expression in quadriceps muscle is equivalent in mice with and without adenine-induced renal impairment among the wild-type groups (Figure 3.12F). While 11βHSD1-KO mice in the control condition show higher Gilz expression compared to 11βHSD1-KO mice with renal impairment, skeletal muscle Gilz expression in the latter group is at a level comparable to wild-type animals (Figure 3.12F).

Liver and adipose tissue are other major sites of 11βHSD1 enzymatic activity. 11βHSD1 activity in liver, measured as ex vivo corticosterone production rate, is slightly reduced but not significantly different in adenine-fed wild-type animals compared to chow-fed controls (relative difference -23.4%, $p>0.05$, Figure 3.13A). A trend for slightly higher 11βHSD1 activity

in adipose tissue of adenine-fed wild-type animals again does not reach statistical significance (relative difference +25.4%, p>0.05, Figure 3.13A). In both tissues, corticosterone production is markedly reduced for 11βHSD1-KO animals (-84.5% and -70% in liver and fat respectively).

Glucocorticoid metabolism and signalling were additionally examined locally within kidney tissue, which is a major site of 11βHSD2 activity and also expresses 11βHSD1 at lower levels. 11βHSD1 mRNA expression is significantly lower in wild-type animals with adenine-induced renal impairment compared to control animals (-3.1x fold change, p<0.0001, Figure 3.13C). Expression of 11βHSD2 in renal tissue is also reduced in adenine-treated animals (diet effect p=0.001, genotype effect p>0.05 & interaction p>0.05 in 2-way ANOVA; Figure 3.13D). Despite these changes, expression of Gilz mRNA as a surrogate marker of glucocorticoid signal transduction locally within kidney tissue does not show any differences for any experimental group following the 7-week 0.15% adenine protocol (Figure 3.13E).

Figure 3.13 Glucocorticoid phenotype in liver, adipose tissue and kidneys of wild-type and 11βHSD1-KO mice receiving normal chow or 0.15% adenine diet.
[figure on following page] ***A-B)*** *11βHSD1 oxoreductase activity in liver and adipose tissue ex vivo. Data from wild-type animals receiving normal chow (n=6), wild-type animals receiving 0.15% adenine diet for 7 weeks (n=9) and from 11βHSD1-KO animals (n=3) respectively.* ***C-E)*** *mRNA expression of Hsd11b1, Hsd11b2 and Gilz in kidney tissue after normal chow or 0.15% adenine diet for 7 weeks. mRNA expression level was determined by rtPCR and normalised to the housekeeper gene 18S.* ***F)*** *11βHSD2 dehydrogenase activity and* ***G)*** *11βHSD1 oxoreductase activity in kidney tissue ex vivo. This data is from a supplementary experiment of wild-type animals receiving normal chow (n=6), wild-type animals receiving 0.15% adenine for the shortened period of 5 weeks (n=4), and 11βHSD1-KO animals (n=2). Analysis by one-way ANOVA with Dunnett's post-hoc test (for A, B, F and G), or by two-way ANOVA with Tukey's multiple comparisons test (for C, D and E); ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001.*


A
11βHSD1 activity (liver)
corticosterone production (fmol/mg/hr)
****
ns
normal chow
0.15% adenine
11βHSD1-KO
B
11βHSD1 activity (adipose tissue)
corticosterone production (fmol/mg/hr)
*
ns
normal chow
0.15% adenine
11βHSD1-KO
C
Hsd11b1 mRNA (kidney)
mRNA expression (AU)
****
****
****
ns
wild-type
11βHSD1-KO
D
Hsd11b2 mRNA (kidney)
mRNA expression (AU)
ns
ns
ns
*
wild-type
11βHSD1-KO
E
Gilz mRNA (kidney)
mRNA expression (AU)
ns
ns
ns
ns
wild-type
11βHSD1-KO
F
11βHSD2 activity (kidney)
DHC production (fmol/mg/hr)
ns
ns
normal chow
0.15% adenine (5w)
11βHSD1-KO
G
11βHSD1 activity (kidney)
corticosterone production (fmol/mg/hr)
*
ns
normal chow
0.15% adenine (5w)
11βHSD1-KO
normal chow
0.15% adenine
11βHSD1-KO control

Ex vivo glucocorticoid metabolism was not measured in kidney tissue in the original animal study. Therefore, enzymatic activity for 11βHSD1 and 11βHSD2 in kidney was assessed in animals from a supplementary experiment. This supplementary experiment included animals receiving 0.15% adenine over 5 weeks instead of 7 weeks. Acknowledging reduced sample sizes for this data, corticosterone inactivation rates and corticosterone activation rates in kidney tissue ex vivo appears similar in control and adenine-fed wild-type animals (relative change for corticosterone inactivation and activation rates +47.5% and +2.8% respectively, $p>0.05$ for both changes, Figure 3.13F&G).

Summing up the comprehensive examination of glucocorticoid metabolism and signalling, there is little evidence for elevated glucocorticoid activation by 11βHSD1 in wild-type mice with adenine-induced renal impairment. Even though mRNA expression of 11βHSD1 in skeletal muscle is elevated, this finding is not corroborated in ex vivo enzymatic activity assays. Circulating corticosterone concentration, glucocorticoid metabolism in other peripheral tissues and surrogate markers of glucocorticoid signal activity in skeletal muscle or kidney are similar in control and adenine-fed wild-type mice. The investigation of glucocorticoid phenotype confirmed effective suppression of 11βHSD1 -mediated glucocorticoid activity in a range of tissues from 11βHSD1-KO animals. Genetic deletion of 11βHSD1 is associated with elevated levels of circulating corticosterone. Otherwise, 11βHSD1 genetic deletion does not substantially alter any impact of adenine-induced renal impairment on glucocorticoid metabolism or signalling.

3.3 Discussion

The presented experiment aimed to evaluate the therapeutic potential of 11βHSD1 blockade to prevent muscle atrophy related to persistent renal impairment *in vivo*. A secondary objective consisted of characterising the accompanying physiological changes in this context. Administration of adenine at 0.15% w/w via diet over 7 weeks successfully produced a mouse model of renal impairment with associated atrophy of skeletal muscles. Furthermore, the data showed equivalent induction of renal impairment in mice with global genetic deletion of 11βHSD1 and wild-type mice. For 11βHSD1-KO mice, there was confirmed abolition 11βHSD1 gene expression and substantially reduced capacity for glucocorticoid activation in multiple tissues ex vivo, including in skeletal muscle tissue. Even though the requisite experimental conditions were achieved, genetic deletion of 11βHSD1 had no effect on the skeletal muscle atrophy phenotype in the examined model. The following discussion will critically examine the experimental approach, interpret the experimental findings in finer detail, consider potential explanations for lack of improvement of skeletal muscle atrophy and acknowledge limitations with this study.

3.3.1 The experimental model met the requirements for addressing the research question

The experimental approach of 0.15% dietary adenine administration over 7 weeks succeeded in inducing persistent renal impairment in male C57Bl/6 mice. The adenine diet model of renal impairment has been widely validated in the literature.[259, 342, 344, 384, 388] Exogenous excess of the purine adenine leads to saturation of the usual metabolic clearance pathway via adenine phosphoribosyl transferase. Excess adenine is instead converted to 2,8-

dihydroxyadenine (2,8-DHA) via xanthine dehydrogenase. 2,8-DHA is concentrated in renal tubules, where it precipitates to form crystals and causes tubular injury, inflammation and fibrosis.[344] The consequences of this renal pathology are evident in the experimental animals from this study. Kidneys from adenine-treated animals exhibited fibrotic changes and loss of normal renal architecture on histology, together with evidence of crystal deposition. These changes in combination with kidney atrophy strongly indicate irreversibility of renal injury in this model. Renal injury was accompanied by reduction in renal filtration function, evidenced by elevated serum creatinine and urea levels. Although these surrogate markers are influenced by lean tissue mass, lower lean tissue mass would reduce rather than falsely elevate these indices. Consistent with the literature, serum creatine kinase and alanine transferase levels did not suggest any direct toxicity to skeletal muscle or liver tissue in this model.[389]) In summary, adenine administration resulted in effective and selective induction of sustained renal impairment in experimental mice.

A skeletal muscle atrophy phenotype accompanied renal impairment for the adenine diet protocol of 0.15% over 7 weeks. Obtaining a model with skeletal muscle atrophy was essential for imitating CKD-related sarcopenia and investigating the research hypothesis that 11βHSD1 blockade prevents muscle atrophy in this context. Animals with renal impairment had 19% lower quadriceps muscle mass and 20% lower soleus mass. Skeletal muscle atrophy was further substantiated by reductions in myofibre size. When this study was originally conceptualised in 2019, literature on the skeletal muscle phenotype of the adenine model had been very limited and optimal experimental parameters for generating an appropriate model of CKD-related sarcopenia were unclear.[384-386] Since then, the literature has grown rapidly,

with many reports of similar or more intense adenine dosing regimens supporting this approach to obtain mouse models of renal impairment with skeletal muscle atrophy.[175, 345, 388, 390-396]

Not all measurements of skeletal muscle phenotype changed in animals receiving 0.15% adenine over 7 weeks in this study. Tibialis anterior muscle mass was preserved. This may not be surprising, acknowledging that not all muscle groups are equally affected in animal models of kidney disease or indeed human CKD.[88, 395] Relative protection of anterior tibialis muscle from atrophy compared to other muscles has been described in other studies, attributing this observation to differences in fibre type composition.[388, 397, 398] Preservation of specific muscle groups may also account for a lack of grip strength reduction in adenine-treated animals in this study. While there are reports of adenine diet in mice reducing tibialis anterior muscle mass and grip strength, these rely on administration of adenine at doses of 0.2% and higher.[175, 390, 396] The conducted experiment thus included animals that had readily detectable skeletal muscle atrophy, with further exacerbation of myopathy in this model calling for higher adenine dosage.

Higher dietary adenine dosage is less well tolerated by mice and causes greater body weight loss. A dietary adenine dose of 0.18% was trialled initially, but 75% of animals reached 20% relative body weight loss within 3 weeks of starting the experimental protocol. Maintaining body weight above this threshold constituted a welfare provision to avoid undue animal suffering. Accordingly, the Animal Project Licence for this study stipulated that severity limits would not exceed the moderate grade. Drastic body weight reduction also risks a shift in the

pathophysiological mechanism for muscle atrophy and wider phenotype, with starvation and cachexia-mediated pathology becoming more prominent than renal impairment-related pathology. When the adenine dose was revised to 0.15%, body weight was more stable and all animals competed the full protocol. The impact of adenine dose on mouse body weight in this study is similar or slightly more severe than expected based on reports in the literature, although those may be subject to reporting biases.[343, 345, 384] To conclude, 0.15% adenine over 7 weeks represented a protocol that was reasonably tolerated by animals without inducing marked cachexia.

Another premise for reliable evaluation of the research hypothesis is equivalence of baseline and adenine-induced renal phenotype between wild-type and 11βHSD1-KO animals. There was an isolated finding of less renal atrophy in 11βHSD1-KO animals than WT animals with adenine treatment. However, all other histological and biochemical renal markers were comparable, arguing against a relevant difference in susceptibility to adenine based on genotype. This is corroborated by a study that has previously characterised the adenine model in 11βHSD1-KO animals.[259] Equally, our study and other reports in the literature found no divergence of skeletal muscle phenotype between 11βHSD1-KO and WT animals at baseline.[329] Hence, divergent baseline skeletal muscle phenotype or susceptibility to adenine diet represent unlikely sources of confounding for interpreting the research outcomes.

3.3.2 Genetic deletion of 11βHSD1 does not improve skeletal muscle atrophy in the examined animal model of renal impairment

The research showed no evidence for improvement of skeletal muscle atrophy with genetic deletion of 11βHSD1 in the examined animal model. In wild-type animals, adenine exposure decreased muscle weights by 19.4% for quadriceps, 20.2% for soleus and 3.3% for tibialis anterior relative to the normal chow group. In comparison, muscle weights of 11βHSD1-KO animals decreased by 16.0% for quadriceps, 19.0% for soleus and 5.7% for tibialis anterior in the adenine condition relative to the control condition. No measures of comparison between WT and 11βHSD1-KO were statistically significant. The analysed muscles are heterogenous in their fibre type composition, ranging from slow oxidative type I/IIa fibre type predominant soleus to fast glycolytic type IIb/IIx fibre-type predominant tibialis anterior.[399, 400] This makes it less likely that the experiment failed to uncover muscle- or fibre type-specific effects. Furthermore, there was no difference in quadriceps muscle fibre size analysis between adenine-treated 11βHSD1-KO and WT animals, corroborating the muscle weight observations. Even though grip strength as a functional measure of muscle strength was not altered in the adenine diet condition, there is again no indication that genetic deletion of 11βHSD1 delivered any benefit in this outcome measure. Finally, there were mild trends for lower expression of catabolic genes Foxo1 and Trim63 in quadriceps muscle of adenine-treated 11βHSD1-KO animals than WT animals, but these were not statistically significant. To summarise, 11βHSD1 genetic knock-out did not improve skeletal muscle atrophy across a range of outcome measures in this mouse model of adenine-induced renal impairment.

3.3.3 Detailed phenotype interpretation

3.3.3.1 Metabolic changes

Exploring the reasons why 11βHSD1 genetic knock-out failed to be effective against muscle atrophy in the investigated adenine mouse model may reveal novel insights. To this end, it is important to consider the physiological changes in the experimental model in further detail, including interrelated metabolic changes, inflammation and glucocorticoid status. Findings from this study will also be compared with findings from similar studies in the literature.

Catabolic and anabolic signalling within skeletal muscle was investigated through real-time PCR of protein metabolism-regulating genes. For the adenine condition in this experiment, there were little to no mRNA expression changes among the tested markers of proteosome and myogenesis activity that would promote muscle atrophy. While certain studies similarly found no significant changes in muscle expression for markers such as myostatin [392] in the adenine model, other studies - often with higher adenine dosages - have reported upregulation of Fbxo32, Trim63 and myostatin.[393, 396] Besides a less pronounced catabolic phenotype at lower adenine dosage, additional explanations for absent changes in the tested markers may include muscle atrophy mediated through unexamined pathways, shifts in unexamined post-translational signalling or transitory changes in markers at an unexamined earlier timepoint. Regardless of the explanation, uncertainty about the precise mechanism driving the muscle atrophy phenotype in the experimental model is a complicating factor in the interpretation of the central findings.

Sarcopenia and muscle atrophy is commonly associated with alterations in metabolic phenotype that reflect underlying insulin resistance.[4, 95] In this animal experiment, genetic deletion of 11βHSD1 had a beneficial effect on glucose tolerance and circulating lipid levels. The area under the curve following an intraperitoneal glucose challenge was lower in 11βHSD1-KO animals than WT animals. This was independent of adenine or normal chow condition. Improved glucose tolerance in mice with the 11βHSD1-KO genotype has been firmly established in the literature, lending credibility to these observations.[259, 328, 329] The postulated explanation for improved glucose tolerance in 11βHSD1-KO mice involves attenuated hepatic gluconeogenesis with downregulation of key gluconeogenic enzymes glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, likely as a result of diminished intrahepatic glucocorticoid signalling. Additionally, loss of 11βHSD1 attenuated the rise in serum cholesterol levels associated with the adenine condition, and enhanced changes in serum triglyceride levels in a beneficial manner. The direction of these changes again corresponds with findings in previous studies.[259] Our data did no show impaired glucose tolerance or elevated serum triglycerides in the adenine condition as described previously.[259] This discrepancy may be explained by less intense adenine dosing in our experimental model, and determination of triglyceride levels in non-fasted samples. Taken together, loss of 11βHSD1 modified the metabolic profile of experimental animals favourably in terms of glucose tolerance and circulating lipids, even though there was no distinct detrimental effect on these parameters with the adenine condition in this experiment.

Assuming the beneficial effects on glucose tolerance and lipid profile indeed reflect improved insulin sensitivity in 11βHSD1-KO animals, it allows for two possible explanations why the

expected anabolic effects of improved insulin sensitivity did not translate into an advantageous muscle phenotype. Firstly, insulin resistance was not a relevant factor in the development of muscle atrophy in the experimental model. Secondly, loss of 11βHSD1 improved insulin resistance in other tissues like liver and fat, but not in muscle. Conclusive discrimination between these hypothetical explanations is not possible with the available information and would require further research.

3.3.3.2 Inflammation

Inflammation is critically entangled with chronic kidney disease, sarcopenia and glucocorticoid signalling.[55, 138, 139, 179, 310] Previous characterisations of the adenine model have highlighted the importance of inflammatory cells and signalling for the manifestation of the renal impairment phenotype.[344, 401] Previous studies have also indicated that inflammatory markers are elevated systemically, although these reports are based on adenine models with doses of 0.2% or higher.[382, 401, 402] The data from this study clearly show upregulation of inflammatory cytokine expression in kidney tissue and hypercellularity in renal interstitium with 0.15% adenine diet over 7 weeks. Measurement of serum IL6 levels however returned values below the limit of quantification. This would suggest confinement of inflammation to the kidneys without significant systemic inflammation, although technical failure of the assay cannot be excluded. When inflammation was investigated in skeletal muscle tissue, no evidence of elevated inflammatory cytokine expression or inflammatory cell infiltrate was apparent. Indirect effects on skeletal muscle from circulating cytokines or activated leucocytes remain a possibility and are difficult to exclude. Notably, the data also show no divergence of inflammation phenotype between WT and 11βHSD1-KO animals. Based

on the available results, inflammation was readily detectable in renal tissue in adenine-fed animals, but there was no major independent inflammatory response in skeletal muscle tissue.

3.3.3.3 Circulating glucocorticoids

Understanding the changes to glucocorticoid metabolism and signalling lies at the heart of interpreting the finding of this animal experiment. Serum corticosterone was quantified as a measure of systemic glucocorticoid exposure, as well as an indicator of a systemic stress response. Adenine-induced renal impairment was not associated with changes in systemic levels of corticosterone in circulation. Even though the literature does not hold directly comparable data, Chapagain et al. describe no changes in plasma corticosterone level with the adenine model of renal impairment in rats.[259] In this sense, the model imitates observations from human CKD that one-off measurements of circulating glucocorticoid levels differ little between people with and without CKD. Serial measurements of serum corticosterone would allow higher resolution of fluctuations and cumulative glucocorticoid exposure, but these were not feasible within the scope of this experiment. As far as it is possible to tell from the available information, circulating glucocorticoid excess therefore appears unlikely to have contributed to muscle atrophy in the adenine model.

In contrast to the renal impairment condition, loss of 11βHSD1 was associated with significantly elevated serum corticosterone concentration in both chow-fed and adenine-fed animals. Elevation in serum corticosterone levels have also been reported in other studies for this genotype.[403, 404] To some extent, activation of the hypothalamic-pituitary-adrenal axis is an expected compensatory response for maintaining circulating glucocorticoid levels in the

absence of peripheral glucocorticoid activation.[333] Nevertheless, higher serum corticosterone levels in 11βHSD1-KO animals than WT comparators suggests supraphysiological or excessive HPA axis activation. The explanation for this is unclear, with a reasonable speculation being attenuated negative feedback mechanisms at the hypothalamus level. Regardless of the explanation, circulating glucocorticoid excess is certainly a factor that may counteract the inhibitory effects on glucocorticoid signalling expected with blocking 11βHSD1 -mediated glucocorticoid activation in peripheral tissues.

3.3.3.4 11βHSD type 1 and type 2

Peripheral glucocorticoid signalling and metabolism was scrutinised in considerable detail in this study to elucidate tissue-specific changes. It is, foremost, glucocorticoid signalling locally within skeletal muscle that determines myopathic changes. Expression of 11βHSD1 mRNA was 1.7-fold higher in wild-type animals with renal impairment compared to control animals. However, higher mRNA expression did not result in higher biological activity of skeletal muscle to generate active corticosterone from inactive 11-DHC ex vivo. Abundance of Gilz mRNA, a gene upregulated by glucocorticoid signal transduction, was also comparable in muscle from wild-type animals with and without renal impairment. This suggests similar glucocorticoid signals locally within skeletal muscle tissue of adenine-fed and chow-fed wild-type mice.

Several processes could explain the discrepancy between 11βHSD1 mRNA upregulation and unchanged 11βHSD1 enzymatic activity. mRNA may not be translated into protein or altered post-translational processing reduces biological activity. Alternatively, the oxidative environment associated with declining renal function and subsequent shifts in NADPH/NADP+

cofactor ratio may antagonise 11βHSD1 oxoreductase activity.[405] Contrarily, changes in 11βHSD2 or H6PD expression within muscle appear less likely to account for the lack of correlation between 11βHSD1 mRNA expression and enzymatic activity. Despite these uncertainties, the conducted animal experiment does not support augmented glucocorticoid signalling mediated via 11βHSD1 locally within skeletal muscle as a major contributor to muscle atrophy in this adenine model of renal impairment.

Glucocorticoid metabolism was additionally investigated in liver, fat and kidney, the sites of major 11βHSD1 and 11βHSD2 expression, to facilitate comprehensive interpretation of the systemic and muscle phenotypes. With adenine administration at 0.15% over 7 weeks, ex vivo 11βHSD1 enzymatic activity decreased mildly in liver tissue and increased mildly in adipose tissue, but neither change was statistically significant. This contrasts with the findings by Chapagain et al, who describe elevated 11βHSD1 activity in liver for a rodent model of renal impairment, albeit in adenine-fed rats rather than mice.[259]

Intriguingly, 11βHSD1 mRNA expression was markedly suppressed in kidneys of adenine-fed mice. This may reflect replacement of cells that express 11βHSD1 with cells that do not express 11βHSD1 as a consequence of adenine-induced renal injury, as local elevation of inflammatory cytokines such as TNFα would otherwise be expected to upregulate 11βHSD1 expression. Consistent with notions from research involving patients with CKD [301], adenine-induced renal injury is also associated with reduced 11βHSD2 mRNA expression in kidney tissue. Balanced downregulation of 11βHSD1 and 11βHSD2 in kidney could account for no substantial changes in renal Gilz expression, ex vivo DHC conversion assay or ex vivo corticosterone

conversion assay in chow-fed versus adenine-fed wild-type animals. As a caveat, it must be acknowledged that data on renal ex vivo 11βHSD1 and 11βHSD2 is preliminary. Moreover, systemic 11-DHC was not measured in this experiment, which would allow inference about systemic balance of glucocorticoid activation and inactivation. Summarising the available findings, wild-type animals with renal impairment exhibited only marginal changes in 11βHSD1 activity in liver and adipose tissue, but there was concomitant reduction of 11βHSD1 and 11βHSD2 expression in adenine-injured kidneys. The lack of meaningful upregulation of 11βHSD1 expression and activity in the renal impairment condition likely reduces the scope for therapeutic benefit of 11βHSD1 blockade in this experimental setting.

Characterisation of the glucocorticoid phenotype demonstrated that genetic deletion of 11βHSD1 successfully diminished glucocorticoid activation in peripheral tissues to a large extent. mRNA expression of 11βHSD1 in muscle and kidney was undetectable in 11βHSD1-KO animals. Biological activity for conversion of 11-DHC to corticosterone was diminished around 85% in muscle, liver and kidney tissue in ex vivo enzymatic assays, and around 70% in adipose tissue. The residual glucocorticoid activation may be due to reverse catalysis by the 11βHSD2 enzyme or non-specific metabolic reactions. In any case, the 11βHSD1-KO mouse provided an effective research instrument for investigating the potential therapeutic effects of 11βHSD1 blockade.

11βHSD1-KO and WT animals exhibited broadly similar patterns for 11βHSD2 expression changes in adenine-injured kidneys, or lack of change in 11βHSD2 expression in skeletal muscle of adenine-fed animals. Gilz mRNA abundance was also equivalent in kidneys of 11βHSD1-KO

and WT animals, regardless of diet condition. In contrast, there was a trend for elevated Gilz expression in skeletal muscle of 11βHSD1-KO mice compared to WT mice in the chow-fed condition, which was no longer evident in the adenine-fed condition. A hypothetical explanation could consist of elevated serum corticosterone augmenting glucocorticoid signalling in skeletal muscle at baseline, while an unknown mechanism reduces glucocorticoid sensitivity in skeletal muscle in the renal impairment condition. Nevertheless, interpretation of an isolated finding calls for prudence and would require further research to investigate.

3.3.4 Consideration of the null hypothesis

The analysis of the available data allows for four possible explanations why genetic deletion of 11βHSD1 was ineffective in improving muscle atrophy in the tested mouse model of renal impairment. Firstly, systemic glucocorticoid levels were not substantially changed in the experimental model of 0.15% adenine administration over 7 weeks. Preclinical and early clinical studies have suggested that 11βHSD1 inhibition is most effective in preventing muscle atrophy in the setting of marked glucocorticoid excess.[329, 332, 339, 351] Whether 11βHSD1 inhibition is also capable of improving muscle phenotype in the absence of gross glucocorticoid excess has not been conclusively established.[263, 340, 404]

Secondly, 11βHSD1 activity in skeletal muscle was not elevated in the experimental model of renal impairment. In the absence of pathological overactivity of 11βHSD1, its contribution to myopathic changes in renal impairment is less clear and the scope for therapeutic benefit is more limited. It has been postulated that accumulation of inflammatory cytokines in renal

impairment upregulate 11βHSD1 in renal disease [259, 320], but the data from this animal study did not support this.

Thirdly, excessive upregulation of the HPA axis and over-compensation for loss of peripheral glucocorticoid activation was observed in 11βHSD1-KO animals, leading to elevated serum corticosterone concentrations compared to wild-type animals. This would counteract inhibitory effects on glucocorticoid signalling in peripheral tissues expected with loss of 11βHSD1 activity. Even though compensatory upregulation of HPA axis activity has also been described in clinical trials of 11βHSD1 inhibitors, supraphysiological levels of cortisol have not been reported in studies lasting up to 12 weeks.[333, 335, 340]

Finally, it is possible that 11βHSD1 may not function predominantly as an oxoreductase in skeletal muscle, but rather act as a bidirectional enzyme.[190] It is fair to acknowledge that most studies, especially studies in mice, characterise 11βHSD1 in skeletal muscle primarily as an oxoreductase.[327, 329, 330] Nevertheless, this hypothesis has been proposed before as a putative explanation for moderate efficacy of 11βHSD1 inhibitors in clinical trials for patients with metabolic diseases.[335]

3.3.5 Limitations

Several limitations should be acknowledged for the animal experiment:

- Research findings based on animal models may not be true for humans and human disease

- The study relied on a single animal model of renal impairment. Alternative models, either with different adenine dosing protocols or different mechanisms of renal injury, may exhibit different inherent characteristics that change the significance of 11βHSD1 for the associated skeletal muscle phenotype [259, 345]

- The study used a global germline deletion of 11βHSD1 as the research instrument for suppressing 11βHSD1 activity. While this was effective, an inducible or muscle tissue-specific inhibition of 11βHSD1 activity could potentially deliver greater therapeutic benefit, as this may avoid or lessen compensatory responses in systemic glucocorticoid homeostasis

- The study was only conducted in male animals. Whether the effects of 11βHSD1 inhibition would be similar or different in female mice remains unclear. The rationale for not extending the study to female animals included the knowledge that female animals are relatively resistant to muscle atrophy in the adenine diet model of renal impairment.[345] The reasons for this are not yet fully understood.

- The study did not investigate the role of androgens. Relative androgen deficiency has been implicated in the aetiology of sarcopenia related to renal disease [26, 147], and 11βHSD1 inhibition modulates androgen signalling.[340] In the first instance, a detailed characterisation of the glucocorticoid phenotype was prioritised. Given the lack of change in skeletal muscle phenotype with loss of 11βHSD1 in mice with renal impairment, further characterisation of the androgen phenotype was not deemed a priority.

- The predominant cause(s) for muscle atrophy in the examined experimental model could not be confirmed. Investigation of the ubiquitin-proteosome pathway, myostatin expression, glucose homeostasis, inflammation and glucocorticoid signalling could not identify a clear mechanism for reduced muscle mass and fibre size in the adenine-fed animals. These aspects were prioritised for investigation based on their close relation to 11βHSD1 and glucocorticoid signalling in previous studies. Yet, other putative atrophic stimuli were not measured in this study, including acidosis, anorexia, inactivity, etc. Knowledge of the underlying myopathic mechanism could provide valuable insights into the likelihood of 11βHSD1 inhibition to succeed or fail as strategy to prevent muscle atrophy.

- The study could not characterise all signalling pathways in skeletal muscle that contribute to muscle atrophy. PI3K-Akt-mTOR signalling, autophagy, myogenesis or ribosomal activity are examples of pathways that were deemed less relevant to the primary research question and outside the scope of this study.

3.3.6 Summary

This animal experiment examined the research question whether blocking 11βHSD1 function prevents muscle atrophy related to persistent renal impairment *in vivo*. The experimental protocol successfully delivered an animal model with verified selective impairment of kidney function and verified accompanying skeletal muscle atrophy. Mice with genetic loss of 11βHSD1 had confirmed loss of glucocorticoid activating capacity in peripheral tissues and developed a comparable adenine-induced renal injury as wild-type animals. Having achieved

all these preconditions, loss of 11βHSD1 function did not alter reductions in muscle weights or fibre size in renal impairment. Putative explanations for the lack of efficacy relate to lack of substantial changes in glucocorticoid signalling or 11βHSD1 activity in the studied experimental model, and adaptations in the HPA axis with loss of 11βHSD1 function.

Chapter IV – In vitro experiments to characterise the regulation of 11βHSD1 in human muscle tissue in response to discrete aspects of renal impairment

4.1 Introduction

4.1.1 Rationale for experimental approach

This second research results chapter discusses cell culture experiments with human muscle cells and conditions mimicking CKD to characterise regulation of 11βHSD1 function and protein metabolism. Cell culture experiments offer a useful approach to refine mechanistic understanding of biomolecular processes. The rationalised and precisely controlled conditions in cell culture experiments permit dissociation from a multitude of physiological and behavioural influences (e.g. diurnal rhythm, nutrition, physical activity, etc.), thereby enabling the investigation of discrete stimuli in isolation. Furthermore, straightforward application of many biomolecular assays to cell cultures facilitates efficient investigation of biomolecular pathways and tissue-specific metabolic activity. As a drawback, the artificial environment for cells in vitro may introduce artefactual phenomena diverging from tissue in situ. Nevertheless, cell culture experiments provide a valuable technique in the toolbox of scientific exploration, especially as a complementary methodology to in vivo and human research.

Muscle cell culture experiments have long been a cornerstone for advancing knowledge on muscle physiology and pathology.[377] This includes insights into atrophic factors related to kidney disease, for example acidosis or myostatin.[249, 256] Yet, the majority of experiments relied on rodent-derived immortalised cell lines. Species differences and genetic mutations bestowing immortalisation present obvious limitations for applying these results to human physiology and disease.[406, 407] Primary human muscle culture techniques have been established.[408-410] Yet, dependence on fresh human muscle biopsies, prolonged culture

periods and limited passage numbers present challenges that limit wide-spread use. Collaborating with Prof Simon Jones has made it possible to access fresh human skeletal muscle tissue on a regular basis for this study. These tissue samples are donated by patients with osteoarthritis during elective joint replacement surgery as part an ongoing study on hormonal and paracrine regulation of skeletal muscle.(IRAS: 206880) Provided with this resource, in vitro experiments in this study exclusively use human primary muscle cells to maximise applicability of results to human physiology and disease.

Skeletal muscle tissue derived from patients with osteoarthritis provide a pertinent platform for studying CKD-associated conditions in vitro. The population with osteoarthritis typically has a comparable age and gender distribution to the CKD population. Yet, muscle tissue donors did not have kidney disease and hence their muscle tissue is naïve to the effects of CKD. To recreate the complex chemical milieu associated with kidney dysfunction in vitro, cells are exposed to serum from patients with CKD, while serum from humans without CKD is used as the control condition. This method aims to mimic CKD-related changes in circulating factors wholistically, incorporating known and unknown shifts in hormones, cytokines, metabolites and other constituents of extracellular fluid. The literature holds several examples of this experimental approach proving effective, e.g. uraemic serum inducing 11βHSD1 expression in human liver cells or serum from donors with liver disease inducing catabolic genes in rodent muscle cells.[324, 411] This experimental set-up therefore offers an apt system for investigating effects of circulating factors associated with human CKD specifically in human skeletal muscle tissue. This approach will be used to explore regulation of 11βHSD1 in muscle cells and additionally explore changes in myocellular protein metabolism.

Certain factors in cell culture are not strongly affected by the type of serum used, but predominantly determined by the properties of the base medium. Such factors include availability of macronutrients, select micronutrients and pH. Given that acidosis is of particular interest when it comes to muscular atrophy and interaction with glucocorticoid signalling in CKD [126, 256], the study includes a separate set of experiments to explore the influence of pH on myocellular 11βHSD1 regulation.

4.1.2 Chapter aim and objectives

The following in vitro experiments will examine the regulation of 11βHSD1 in human skeletal muscle cells in response to serum from patients with CKD, or in response to acidosis. Subsequently, the experimental system of human muscle cells exposed to CKD serum will be used to investigate influences on myocellular protein synthesis and degradation.

Aim

2. Characterise the regulation of 11βHSD1 and its functional impact on myocellular protein metabolism under CKD-associated conditions in human primary muscle culture.

Objectives

a) Verify the authenticity of human primary muscle cell cultures and their capacity for dynamic regulation of 11βHSD1
b) Examine the effect of serum from patients with CKD on regulation of 11βHSD1 in human primary muscle cultures

c) Examine the effect of acidosis on regulation of 11βHSD1 in human primary muscle cultures

d) Examine the effect of serum from patients with CKD on myocellular protein metabolism in human primary muscle cultures

4.2 Results

4.2.1 Validation of muscle cell culture protocol and inflammatory regulation of 11βHSD1

Primary human muscle cell cultures were used as an experimental system to explore regulation of 11βHSD1 in skeletal muscle by CKD-associated factors in greater mechanistic detail. In the beginning, several checks were carried out to confirm the adequacy of the cell culture protocol and validity of this experimental system. Upon changing cell culture conditions from proliferation media to differentiation media, expression of myoblast markers Myf5 and Myf6 decreases swiftly between day 0 and day 4 (Figure 4.1). Expression of MyoD also declines more gradually over 8 days. The muscle differentiation gene MyoG is transiently upregulated between day 0 and day 4, before being downregulated again from day 4 to day 8. This profile of gene expression changes is consistent with a differentiation transition from myoblasts to myotubes.[412] Phase contrast microscopy shows a change from cells with a fusiform appearance at day 0 to cells with tubular structure at day 8 (Figure 4.2). Immunofluorescence microscopy with staining for the muscle cell differentiation marker desmin further confirms successful maturation of cultured muscle cells from day 0 to day 8. These observations from gene expression profiles, cellular appearance and positive desmin staining together suggest that the experimental protocol is effective for yielding differentiated human skeletal muscle cells.

As the next step, the fundamental properties of 11βHSD1 enzymatic activity in this cell culture system were characterised and validated. 11βHSD1 enzymatic activity, measured as in vitro conversion rate of cortisone to cortisol, is substantially higher in differentiated myotubes than

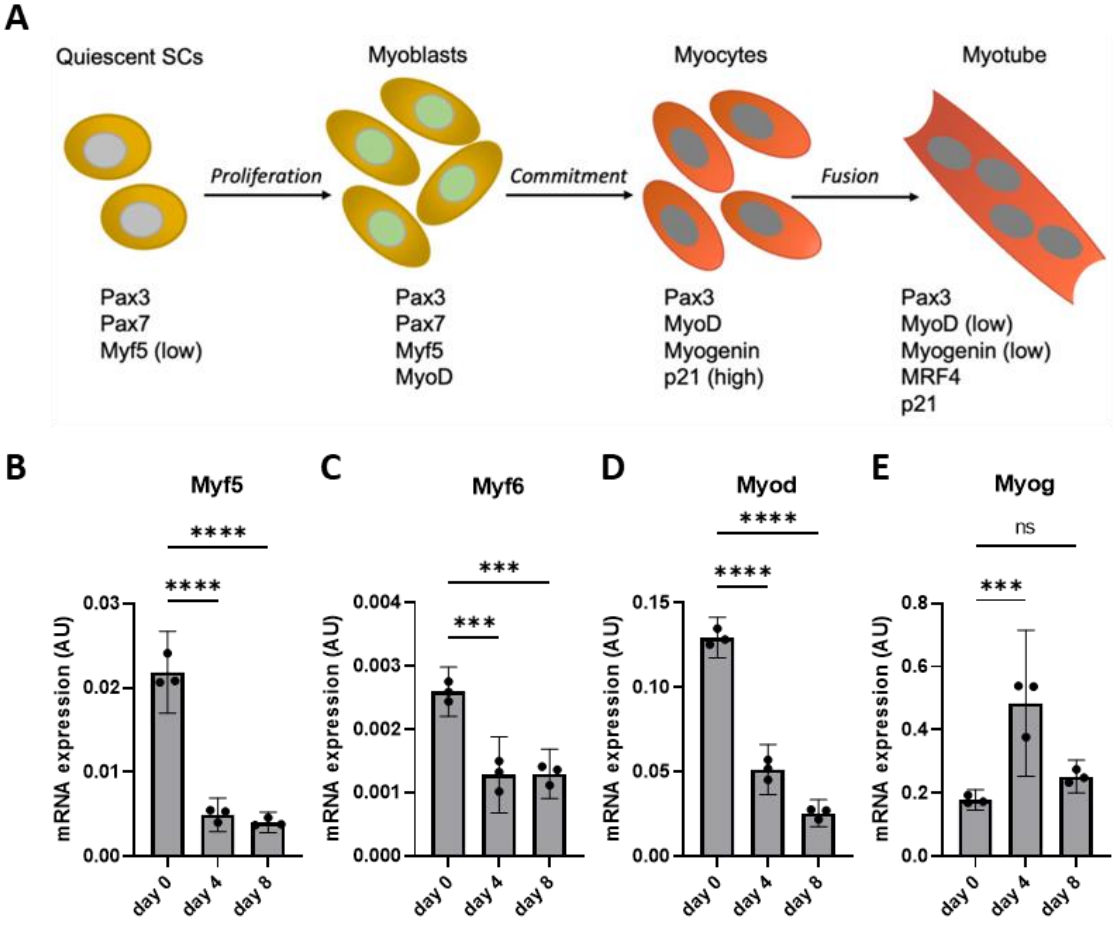


Figure 4.1 mRNA expression dynamics of selected muscle differentiation genes during transition of human primary myoblasts to myotubes in vitro.

A) *Diagram of muscle cell differentiation pathway from stem cells (SCs) to multinucleated myotubes and corresponding expression profiles of genes involved in myogenesis. Diagram with annotations reproduced from [412]* ***B-E)*** *mRNA expression of selected myogenesis genes during the in vitro protocol for differentiating primary human myoblasts into myotubes. Myoblasts at 70-80% confluence between passages 2-4 were switched from proliferation media (day 0) and maintained in differentiation for 8 days. mRNA was isolated in triplicate from the same primary human muscle cell lineage at day 0, day 4 and day 8 of the in vitro differentiation protocol. mRNA expression was quantified by rtPCR and normalised to Gapdh. Analysis by one-way ANOVA with Dunnett's multiple comparisons test; ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

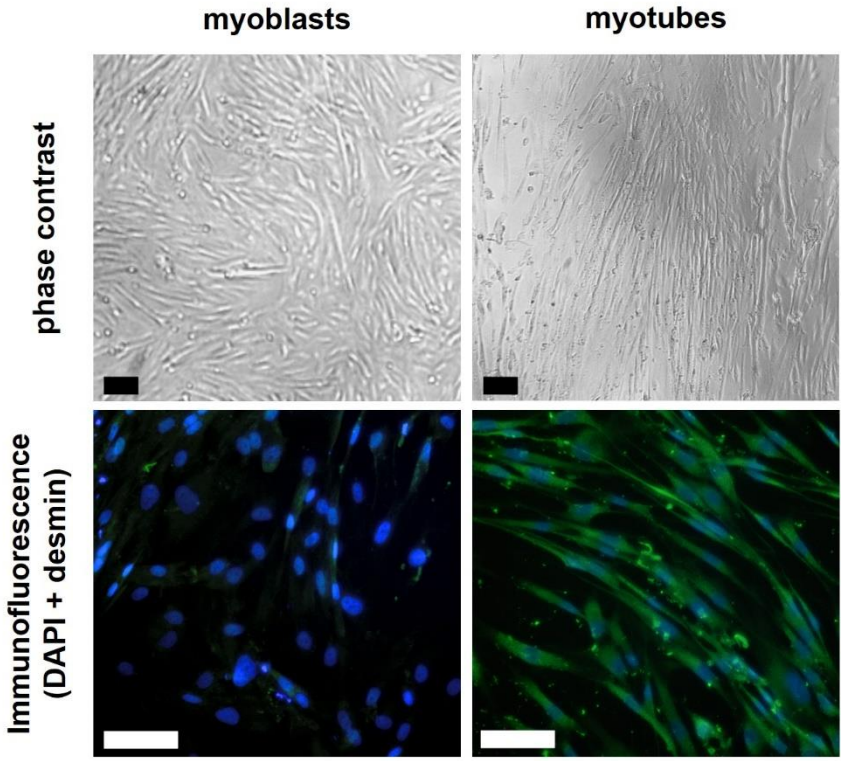


Figure 4.2 ***Microscopy of human primary muscle cell cultures.***
A-B) *Phase contrast light microscopy of undifferentiated proliferating myoblasts and differentiated myotubes, magnification x100.* ***C-D)*** *Immunofluorescence confocal microscopy of myoblasts and myotubes with staining for nuclei with DAPI (blue) and for the muscle cell differentiation marker desmin (green), magnification x200. Inset scale bars indicate 100microns.*

undifferentiated myoblasts (24.3±2.7pmol/mg protein/h and 4.0±1.5pmol/mg protein respectively, $p<0.001$, Figure 4.3A). A time-course of cumulative cortisone-to-cortisol conversion demonstrates that enzymatic rate remains stable over an assay period up to 30 hours. Furthermore, the time-course experiment illustrates clearly that pre-treatment with TNFα augments 11βHSD1 enzymatic rate and co-incubation with the inhibitor glycyrrhetinic acid suppresses enzymatic activity (Figure 4.3B). rtPCR confirms that the pro-inflammatory cytokine TNFα upregulates expression of 11βHSD1 in cultured muscle cells (14.5x fold change, $p=0.023$, Figure 4.3C).

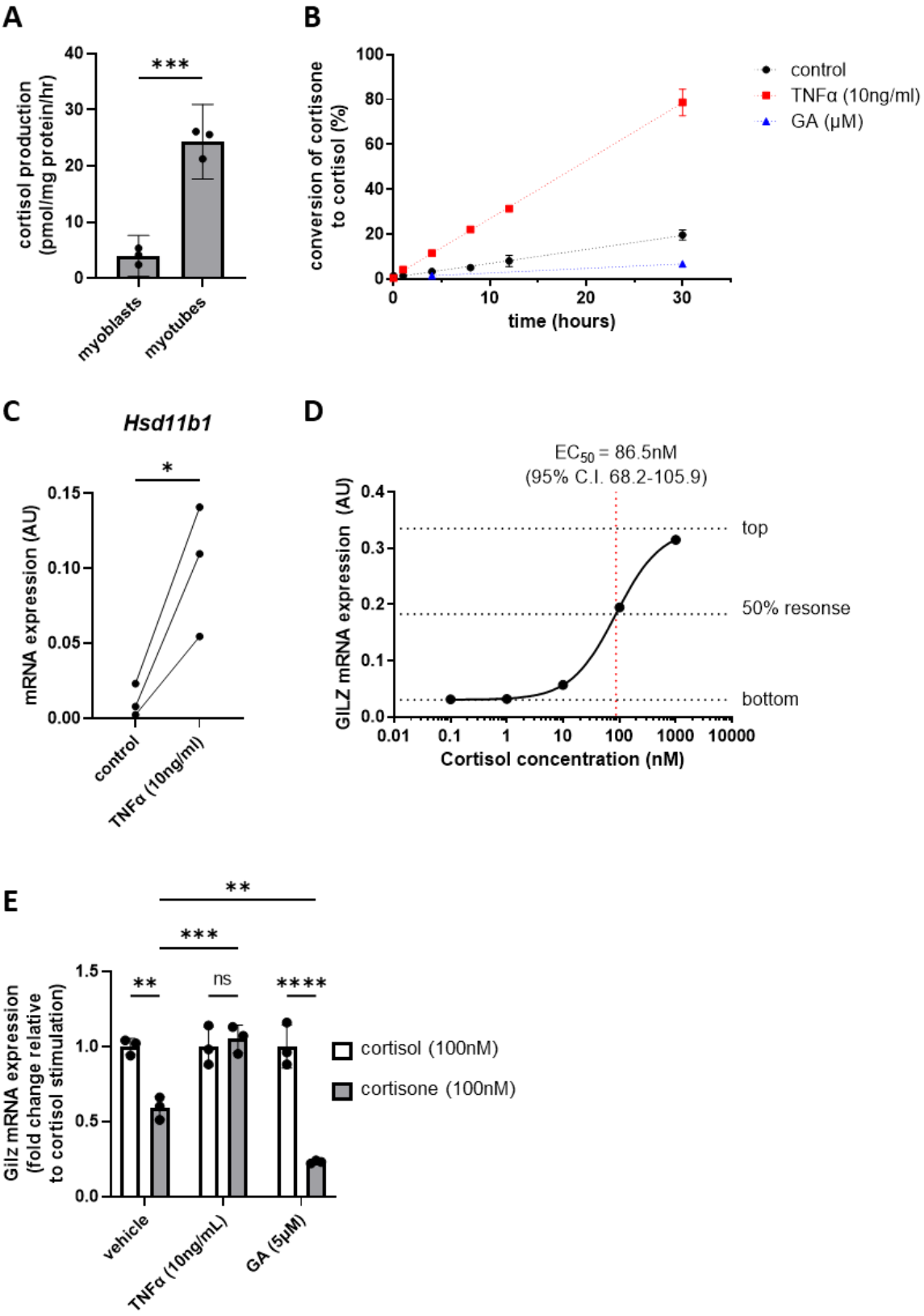

A
cortisol production (pmol/mg protein/hr)
***
myoblasts
myotubes
B
conversion of cortisone to cortisol (%)
time (hours)
control
TNFα (10ng/ml)
GA (μM)
C
Hsd11b1
mRNA expression (AU)
*
control
TNFα (10ng/ml)
D
EC50 = 86.5nM
(95% C.I. 68.2-105.9)
GILZ mRNA expression (AU)
top
50% resonse
bottom
Cortisol concentration (nM)
E
Gilz mRNA expression (fold change relative to cortisol stimulation)
**
***
ns
****
cortisol (100nM)
cortisone (100nM)
vehicle
TNFα (10ng/mL)
GA (5μM)

Figure 4.3 Characterisation of 11βHSD1 enzymatic activity in primary human muscle cells.

[figure on previous page] ***A)*** *Enzymatic production rate of active cortisol from inactive cortisone by 11βHSD1 in undifferentiated myoblasts and differentiated myotubes in vitro. Experiment performed in triplicate with cultures from one primary human muscle cell lineage. Analysis by unpaired t-test.* ***B)*** *Time course for fractional conversion of cortisone to cortisol by human myotubes in vitro in the presence of standard differentiation media (control / black), differentiation media plus TNFα (TNF / red; treatment applied for 24h before assay) and differentiation media plus 11βHSD1 inhibitor glycyrrhetinic acid (GA / blue). Experiment performed in duplicate with cultures from one primary human muscle cell lineage. Points indicate means, error bars indicate standard deviation and dotted line indicates linear regression fit.* ***C)*** *mRNA expression of 11βHSD1 gene in myotubes at baseline and following stimulation with TNFα at 10ng/ml for 24h. Experiment performed in cell cultures from three distinct lineages derived from three separate muscle tissue donors. Analysis by paired t-test.* ***D)*** *Dose response curve for induction of Gilz mRNA expression after stimulation with cortisol for 24 hours. Curve fit and interpolation of* EC_{50} *by least squares regression with four parameter model using GraphPad Prism (version 9.5).* ***E)*** *Gilz mRNA expression response with cortisone treatment at 100nM for 24h normalised to the Gilz mRNA expression level with cortisol treatment at 100nM for 24h in the respective conditions of vehicle, TNFα co-treatment and glycyrrhetinic acid (GA) co-treatment. Experiment performed in triplicate with cultures from one primary human muscle cell lineage. Analysis by two-way ANOVA with Tukey's multiple comparisons test; ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

Gilz gene expression is regulated by the activated glucocorticoid receptor and is a useful surrogate marker of glucocorticoid signalling.[413, 414] To validate Gilz expression as a surrogate marker of glucocorticoid stimulation in cultured muscle cells, a dose-response experiment was performed. This shows dose-dependent upregulation of Gilz mRNA expression over a cortisol concentration range from 1nM to 1000nM, with an estimated EC_{50} = 86.5nM (95% C.I. 68.2-105.9) (Figure 4.3D). When muscle cells are stimulated with either cortisol or cortisone at 100nM for 24h, Gilz expression is 41.0% less with cortisone than with cortisol, indicating partial conversion of inactive cortisone to active cortisol (-1.7x fold change,

p=0.002, Figure 4.3E). In comparison, pre-treatment with TNFα eliminates a differential response in Gilz expression between cortisol and cortisone (1.05x fold change, p>0.05), whereas co-incubation with glycyrrhetinic acid expands the differential reponse (-4.3x fold change, p<0.0001, Figure 4.3E). Taken together, these data varify that cultured human skeletal muscle cells have capacity for 11βHSD1-mediated conversion of cortisone to cortisol, have capacity for dynamic regulation of 11βHSD1 expression and activity in response to TNFα, and have capacity to regulate Gilz expression in a manner that reflects the availability of active glucocorticoid and glucocorticoid signal transduction.

4.2.2 CKD serum does not alter 11βHSD1 activity in human muscle cells

Human muscle cell cultures were stimulated with human serum from donors with or without CKD. This was done to explore effects of any known or unknown CKD-associated circulating factors on 11βHSD1 activity in skeletal muscle. The defining clinical characteristics of healthy volunteers and patients with CKD who provided serum samples for this experiment are summarised in Table 4.1. Serum donors with CKD and healthy donors are matched for age and gender. There is a trend for higher body-mass-index (BMI) and greater prevalence of diabetes among patients with CKD. As intended, healthy donors and patients with CKD have markedly different levels of kidney function, measured as estimated glomerular filtration rate (eGFR). Furthermore, patients with CKD had higher levels of C-reactive protein and HbA1c, indicating higher levels of systemic inflammation and worse glycaemic control. Hence, serum donors are matched for age and gender, but are clearly separated for kidney function and CKD-associated changes like inflammation and glycaemic control.

Table 4.1: Clinical characteristics of serum donors with or without CKD

	Healthy	CKD	p-value
n	5	6	
Age (years)	73.8 ± 5.5	77.7 ± 7.1	n.s.
Female (%)	60%	50%	n.s.
BMI (kg/m^2)	28.6 ± 4.1*	33.2 ± 2.9	n.s.
Diabetes (%)	0%	50%	n.s.
eGFR (mL/min/1.73^2)	78.2 ± 5.9	24.5 ± 6.7	<0.001
ACR (mg/g)	0.5 ± 0.3*	88.7 ± 152.4	n.s.
CRP (mg/L)	0.6 ± 0.5	7.2 ± 3.1	0.003
HbA1c (mmol/mol)	35.2 ± 2.9	49.7 ± 7.6	0.003

*Data shown as mean ± standard deviation, or percentage. Comparisons by unpaired t-test or Fisher's exact test. *no data for 2 healthy subjects. BMI, body mass index; eGFR, estimated glomerular filtration rate; ACR, albumin creatinine ratio in urine; CRP, C-reactive protein.*

Myotubes were treated with 10% or 20% human serum for 48 hours before measuring 11βHSD1 activity. To obtain sufficient serum volume for stimulation at 20%, serum samples from multiple human donors were pooled. Serum concentrations and incubation periods were based on previously published data of dose and time response patterns.[263, 324] Myotube cultures from four different muscle tissue donors were used in this experiment to reduce the risk of unique genetic or epigenetic traits masking potential serum-related effects. Regardless of serum concentration or myotube lineage, 11βHSD1 activity in myotubes exposed to CKD serum is not different to the control condition of healthy serum exposure (10% serum treatment: mean relative difference -5.5% for CKD serum compared to healthy serum, p>0.05 for serum effect, Figure 4.4A; 20% serum treatment: mean relative difference -7.3% and +6.1% for non-diabetic CKD and diabetic CKD serum respectively compared to healthy serum, p>0.05 for both comparisons, Figure 4.4B). In a post-hoc analysis of experiments using 10% serum, there were no significant differences in 11βHSD1 activity with serum from CKD patients with

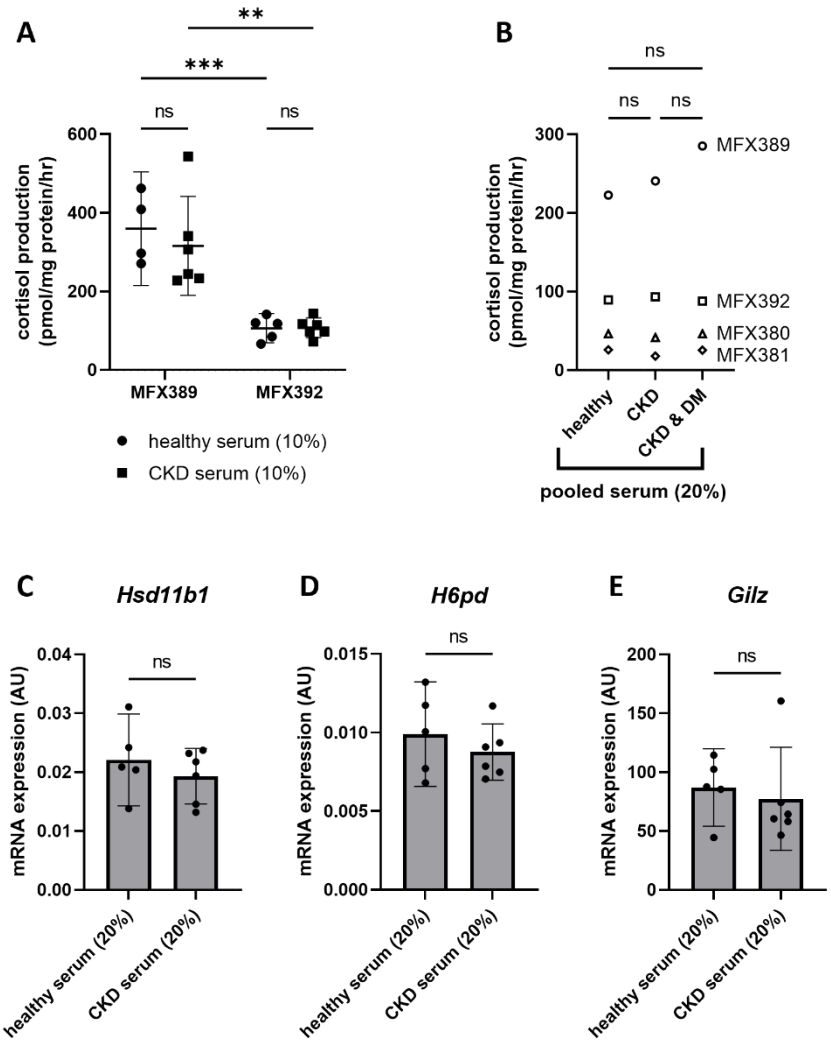


Figure 4.4 Effects of human serum from patients with CKD or healthy volunteers on 11ϐHSD1 activity in human primary myotubes.

A) *11ϐHSD1 activity in human myotubes after treatment with 10% human serum from individual healthy donors (n=5) or patients with CKD (n=6) for 48h. The experiment was repeated in cell cultures from two distinct lineages derived from two separate muscle tissue donors (MFX389 & MFX392). Analysis by two-way ANOVA with Tukey's multiple comparisons test.* ***B)*** *11ϐHSD1 activity in human myotubes after treatment with 20% pooled human serum from healthy donors (n=5), patients with CKD (n=3), patients with CKD and diabetes (DM) (n=3) for 48h. The experiment was repeated in cell cultures from four distinct lineages derived from four separate muscle tissue donors (represented by distinct symbols). Analysis by repeated-measures one-way ANOVA with Tukey's post-hoc test.* ***C-E)*** *mRNA expression of 11ϐHSD1, H6PD and GILZ genes in human myotubes after treatment with 20% human CKD or control serum for 24h. mRNA experiments used muscle cell cultures derived from one single muscle tissue donor (MFX392) treated with individual serum samples from healthy serum donors (n=5) or serum donors with CKD (n=6). Analysis by unpaired t-test; ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

or without diabetes, neither between these subgroups nor compared to healthy serum. Expression of mRNA for 11βHSD1, H6pd and Gilz genes is also similar in myotubes treated with 20% healthy serum or CKD serum (Figure 4.4C-E). Hence, there is no indication that CKD serum alters 11βHSD1 expression or activity relative to healthy human serum in skeletal muscle in the tested experimental conditions.

Inflammatory cytokines regulate 11βHSD1 activity in skeletal muscle and are elevated in serum from people with CKD compared to healthy people.[55, 138, 263, 351] A closer examination of inflammatory mediators was therefore executed to provide additional information about the findings from the previous experiment. Cultured human muscle cells mount a pro-inflammatory response to stimulation with TNFα (10ng/ml), as illustrated by upregulation of Il6 mRNA expression (10.6x fold change, p=0.008, Figure 4.5A). High-sensitivity C-reactive protein is elevated in CKD serum samples compared to healthy serum samples, reflecting higher levels of systemic inflammation in patients with CKD (7.20±3.11mg/L vs 0.53±0.52mg/L, p=0.001, Figure 4.5B). Nevertheless, when myotubes are exposed to 20% CKD or healthy serum, no differential response in mRNA expression of pro-inflammatory cytokines Il6, Il1β or TNFα is evident (Figure 4.5C-E). Similarly, secretion of Il6 protein into media does not differ between myotubes treated with healthy serum or CKD serum (mean percentage difference -0.6% and -22.2% for non-diabetic CKD and diabetic CKD serum respectively compared to healthy serum, p>0.05 for both comparisons, Figure 4.5F). These results suggest that serum from donors with CKD does not elicit greater inflammatory responses in cultured human myotubes than serum from donors without CKD in the examined experimental conditions, despite elevated markers of systemic inflammation in CKD.

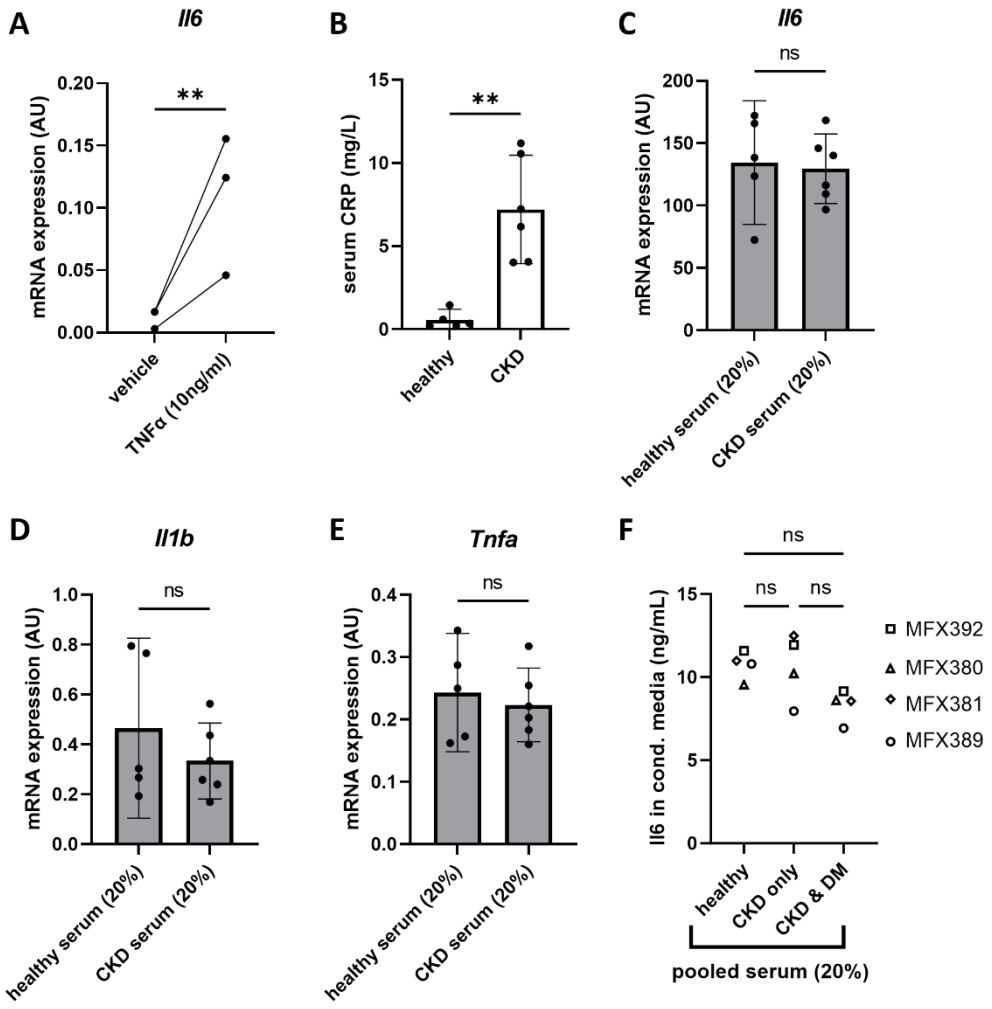


Figure 4.5 Effects of human serum from patients with CKD or healthy volunteers on inflammation markers in human primary myotubes.

A) *mRNA expression of the Il6 gene in human myotubes at baseline and following stimulation with TNFα at 10ng/ml for 24h. Experiment performed in cell cultures from three distinct lineages derived from three separate muscle tissue donors. Analysis by paired t-test.* ***B)*** *High-sensitivity C-reactive protein (CRP) concentration in serum from patients with CKD and healthy volunteers; analysis by unpaired t-test.* ***C-E)*** *mRNA expression of Il6, Il1β and TNFα genes in human myotubes after treatment with 20% human serum from individual healthy donors (n=5) or patients with CKD (n=6) for 24h. mRNA data from muscle cell cultures with the same lineage derived from one single muscle tissue donor. Analysis by unpaired t-test.* ***F)*** *Il6 protein concentration in conditioned media from human myotubes after treatment with 20% pooled human serum from healthy donors (n=5), patients with CKD (n=3), patients with CKD and diabetes (DM) (n=3) for 48h. The experiment was repeated in cell cultures from four distinct lineages derived from four separate muscle tissue donors (represented by distinct symbols). Analysis by repeated-measures one-way ANOVA with Tukey's post-hoc test; ns p>0.05, ** p<0.01.*

4.2.3 Acidosis has little to no effect on 11βHSD1 activity in human muscle cells

It is possible that certain CKD-associated circulating factors are eliminated when serum is diluted in cell culture media. This is the case for metabolic acidosis, which is abolished by the buffer system in cell culture media. Previous studies have established acidosis as an important muscle atrophic factor that requires glucocorticoid signalling as a co-factor.[126, 256] Further experiments were therefore performed to test specifically whether acidosis influences 11βHSD1 activity in cultured human muscle cells.

pH in culture media is usually stabilised with a bicarbonate-based buffer and 5% atmospheric CO2 inside tissue culture incubators. Initially, a validation experiment was performed to confirm that target pH could be stably maintained over the experimental period. Titrating media to target pH is associated with a noticeable colour change, reflecting the properties of phenol red as a pH indicator (Figure 4.6A&B). Media pH measurements after 48 hours in the tissue incubator confirmed that pH is maintained within ±0.1 pH points of the target pH (Table 4.2). Media exposed to cells has a slightly lower pH after 48 hours than media incubated in cell-free tissue culture plates. Cells were stained with Trypan Blue after incubation in acidified media to exclude undue toxic effects. Cells incubated at pH 7.1 have comparable staining to cells maintained at pH 7.4, whereas brief exposure to pH <5 substantially increases Trypan Blue staining (Figure 4.6C). Finally, glucose disposal was measured as a marker of cellular response to acidosis. Upon stimulation with insulin, myotube cultures have lower glucose disposal at pH 7.1 compared to pH 7.4 (mean relative change -28.6%, p=0.032, Figure 4.6D). Taken together, these data illustrate that human primary myotubes can be maintained in stable acidotic culture conditions without undue toxicity and exhibit altered metabolism at lower pH.

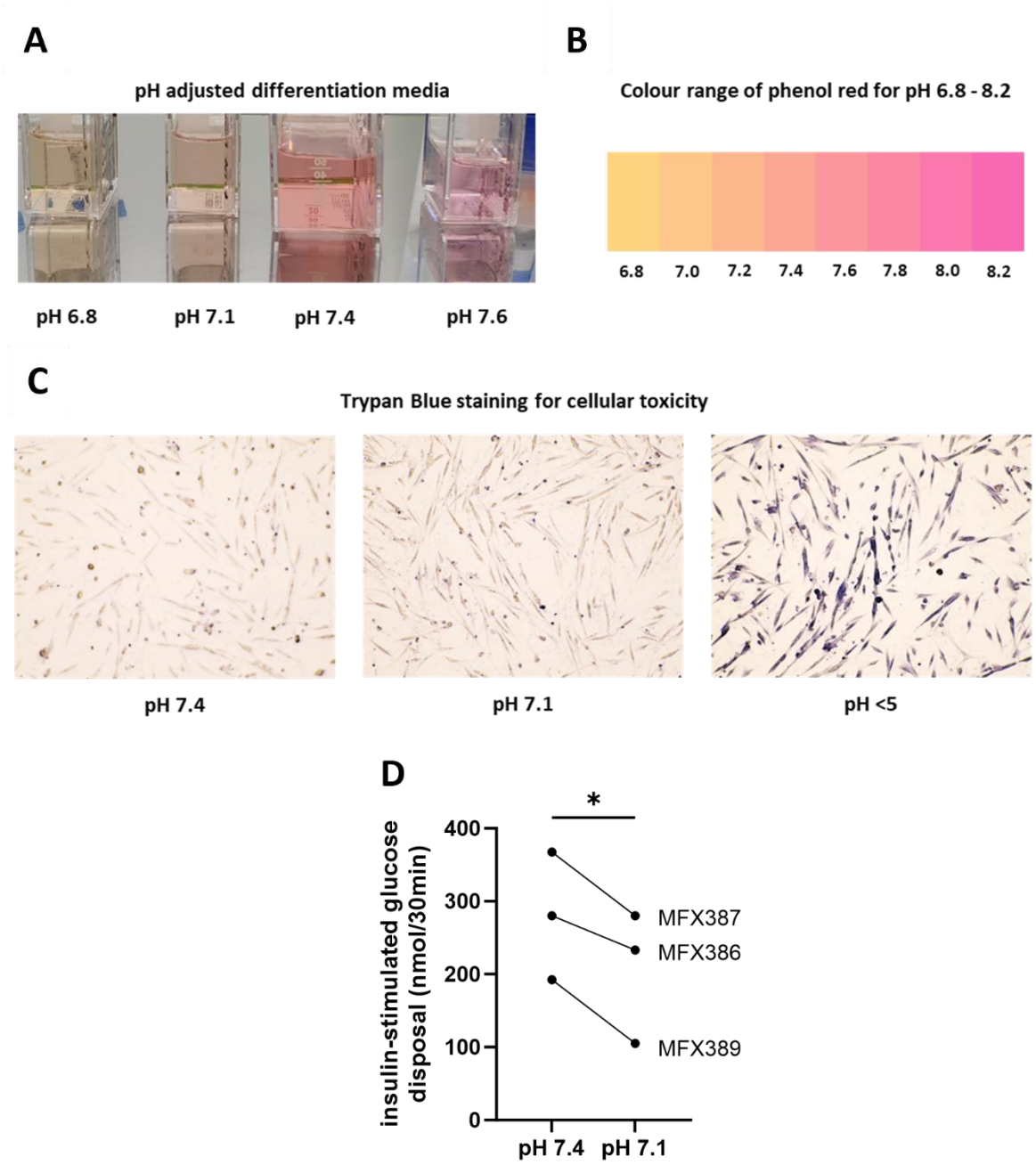


Figure 4.6 Validation of acidosis conditions for cell culture.

A) *Image of colour changes in cell culture media containing phenol red at different pH values.* ***B)*** *Reference colour range of phenol red for pH 6.8 – 8.2. Diagram reproduced from [415]* ***C)*** *Light microscopy of myotubes exposed to acidified culture media and stained with the cellular toxicity indicator Trypan Blue; magnification 100x.* ***D)*** *Insulin-stimulated glucose depletion rate from cell culture media of human primary myotubes at media pH 7.4 and pH 7.1 (insulin at 100nM for 30min). The experiment was repeated in cell cultures from three distinct lineages derived from three separate muscle tissue donors. Comparison by paired t-test; * p<0.05.*

Table 4.2: pH of media before and after storage in cell culture incubator

Experiment	pH target	Measured before incubation	After 48h in incubator (cell-free wells)	After 48h in incubator (with cells)
Validation experiment	7.1	7.07	7.16	7.03
	7.4	7.34	7.46	7.37
11βHSD1 activity experiment	6.8	6.77	6.96	[media used for 11βHSD1 activity assay]
	7.1	7.07	7.22	
	7.4	7.35	7.47	
	7.7	7.62	7.72	

The influence of pH on 11βHSD1 in cultured human myotubes was systematically explored in several experiments. To begin with, acute (no pre-treatment) and prolonged acidosis (48h pre-treatment) at pH 7.1 was tested to explore the relevance of exposure duration for any effect on 11βHSD1. Neither condition leads to a statistically significant change in 11βHSD1 enzymatic activity compared to the control condition at pH 7.4 (relative change -1.8% and -10.3% for acute pH 7.1 and prolonged pH 7.1 respectively compared to pH 7.4, $p>0.05$ for both comparisons, Figure 4.7A), although a trend for reduced 11βHSD1 activity can be observed at pH 7.1 with 48h pre-treatment. To investigate this further, a subsequent experiment conducted in myotubes from four distinct lineages tested a wide range of pH from 6.8 to 7.6 with 48h pre-treatment. This reveals a small but statistically significant reduction of 11βHSD1 enzymatic activity at pH 7.1 and pH 6.8 relative to the control condition at pH 7.4 (relative change compared to pH 7.4: -13.3% for pH 6.8, $p=0.046$; -11.9% for pH 7.1, $p=0.035$, +3.4% for pH 7.6, $p>0.05$; Figure 4.7B). This reduction in enzymatic activity is not associated with reduced mRNA expression of 11βHSD1 or the co-factor supplying H6pd in acidosis conditions

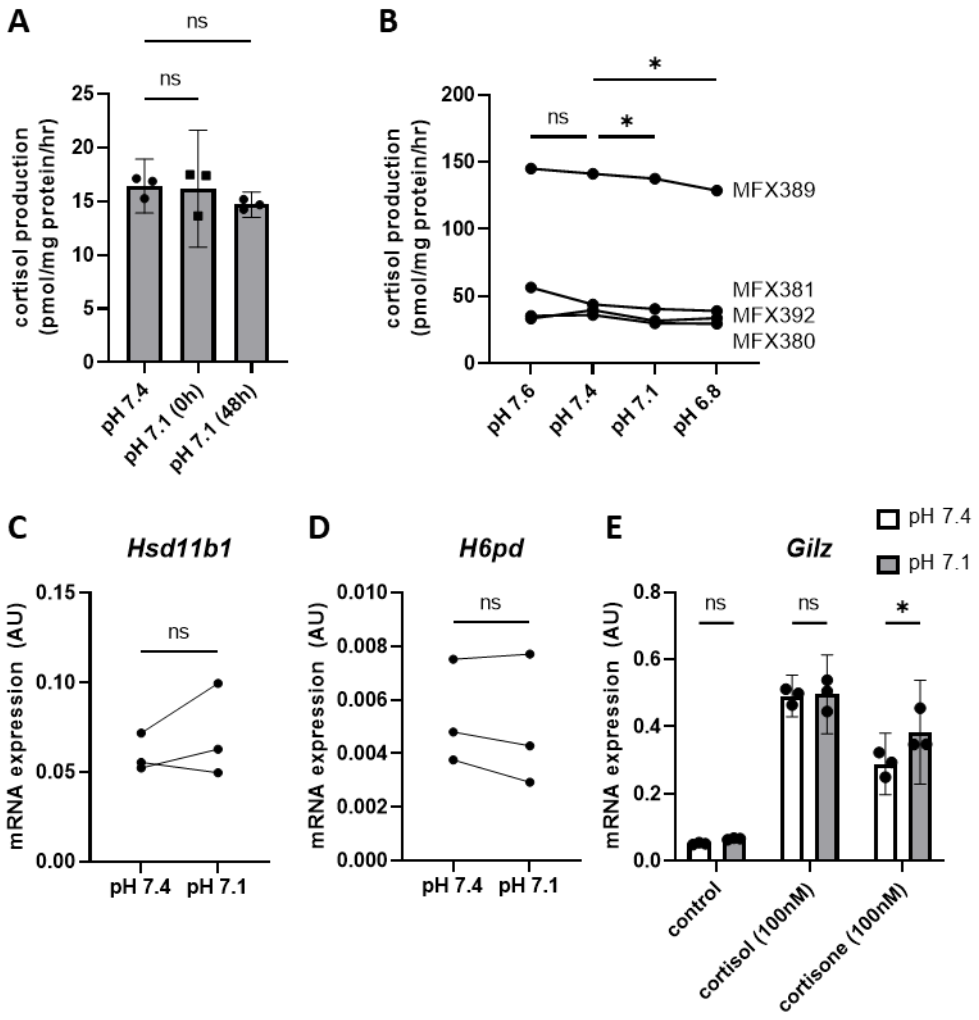


Figure 4.7 Effects of acidosis on 11ϐHSD1 activity in human primary myotubes.

A) *11ϐHSD1 activity of human myotubes at standard conditions with pH 7.4, at pH 7.1 without pre-treatment and at pH 7.1 with 48h pre-treatment. Experiment performed in triplicate with cultures from one primary human muscle cell lineage. Analysis by one-way ANOVA with Dunnett's multiple comparisons test.* ***B)*** *11ϐHSD1 activity of human myotubes after 48h pre-treatment in pH-adjusted differentiation media. Experiment repeated in cell cultures from four distinct lineages derived from four separate muscle tissue donors. Analysis by repeated-measures one-way ANOVA with Dunnett's multiple comparisons test.* ***C-D)*** *mRNA expression of 11ϐHSD1 and H6PD genes in human myotubes after treatment with standard or acidified differentiation media for 48h. Experiment repeated in cell cultures from three distinct lineages derived from three separate muscle tissue donors. Comparisons by paired t-test.* ***E)*** *mRNA expression of Gilz gene in human myotubes treated with standard or acidified media in the absence of additional treatments (control), with added cortisol or with added cortisone for 24h. Experiment performed in triplicate with cultures from one primary human muscle cell lineage. Analysis by two-way ANOVA with Sidak's multiple comparisons test; ns p>0.05, * p<0.05.*

(Figure 4.7C&D). Given the relatively small magnitude of the change in enzymatic activity in acidosis conditions, effects on Gilz expression were investigated to corroborate the significance of this finding. Cortisol signal transduction as measured by upregulation of Gilz mRNA expression is not altered by a pH shift from 7.4 to 7.1 (fold change 1.01x, $p>0.05$, Figure 4.7E). Unexpectedly, Gilz expression in response to cortisone exposure is marginally but significantly higher at pH 7.1 compared to pH 7.4 (fold change 1.33x, $p=0.025$, Figure 4.7E). Based on these results, acidosis does not appear to have a major influence on 11βHSD1 function in skeletal muscle cells, with the conducted experiments yielding equivocal results for marginal changes in 11βHSD1 function.

4.2.4 Acute CKD serum stimulation has equivocal effects on protein metabolism in cultured human muscle cells

As a supplementary line of investigation using the human muscle cell culture system, the effect of CKD serum on protein turnover was explored. Previous studies have suggested that serum from humans with liver disease alters protein metabolism in rodent-derived muscle cell cultures.[411] To the best of our knowledge, this approach has not yet been replicated in human-derived muscle cells. Establishing an in vitro model of catabolic and anti-anabolic effects using human serum and human muscle cells may prove a useful experimental tool to identify unknown circulating factors that influence muscle protein balance and to unravel mechanisms in the pathophysiology of sarcopenia.

The first step consisted of validating the assays for measuring protein synthesis rate and protein degradation rate in cultured human myotubes. Given that the anti-anabolic and

catabolic effects of glucocorticoids are well established, cortisol at 100nM and 1000nM was used as the initial test conditions. Indeed, cortisol stimulates a measurable decrease in global protein synthesis rate and a measurable increase in global protein degradation rate in human myotubes in vitro (Figure 4.8A&B). In a subsequent pilot experiment, human myotubes were exposed to serum from patients with non-alcoholic fatty liver disease (NAFLD) at a concentration of 5% over 24 hours. No significant difference is apparent for protein synthesis rate (33.8±7.8CPM/h/µg protein and 31.0±6.88CPM/h/µg protein for healthy and NAFLD serum respectively, $p>0.05$, Figure 4.8C). For protein degradation rate, NAFLD serum significantly increases protein degradation compared to healthy serum (17.5±1.3CPM%/24h 19.7±0.4CPM%/24h for healthy and NAFLD serum respectively, $p=0.016$, Figure 4.8D). Data from initial characterisation studies therefore show that protein synthesis and degradation rates are responsive to external stimuli in human cultured myotubes. Furthermore, an experimental system involving human muscle cells stimulated acutely with human serum for 24 hours has the potential to induce changes in protein metabolism.

Following on from these initial results, a more systematic investigation of CKD serum effects on protein metabolism in human myotubes was executed. A range of serum concentrations at 2%, 6% and 20% were tested to cover phenotypic effects that may only emerge at higher or lower serum concentration. On examination of protein synthesis rate, no differences are detectable for CKD serum compared to healthy control serum at any tested serum concentration (mean difference 0.36 CPM/h/µg protein, 0.39 CPM/h/µg protein and 0.02 CPM/h/µg protein at 2%, 6% and 20% serum respectively, $p>0.05$ for all comparisons, Figure 4.9A). The control condition with the ribosome inhibitor cycloheximide markedly suppresses

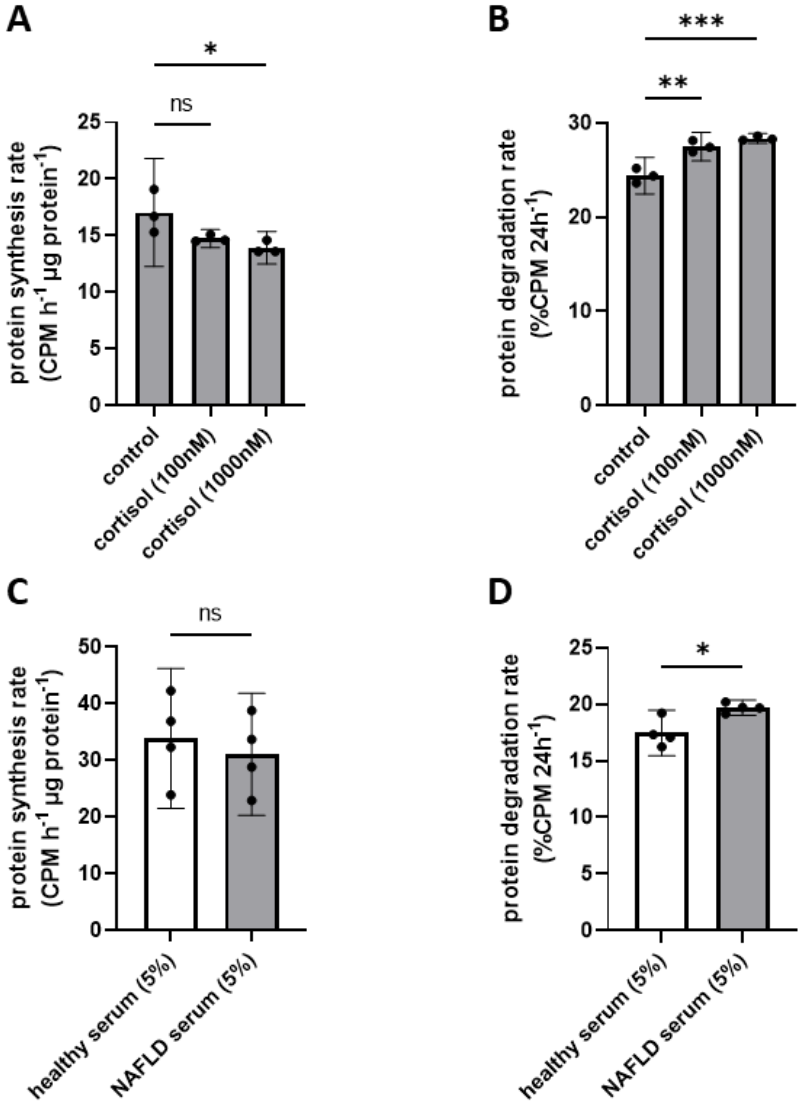
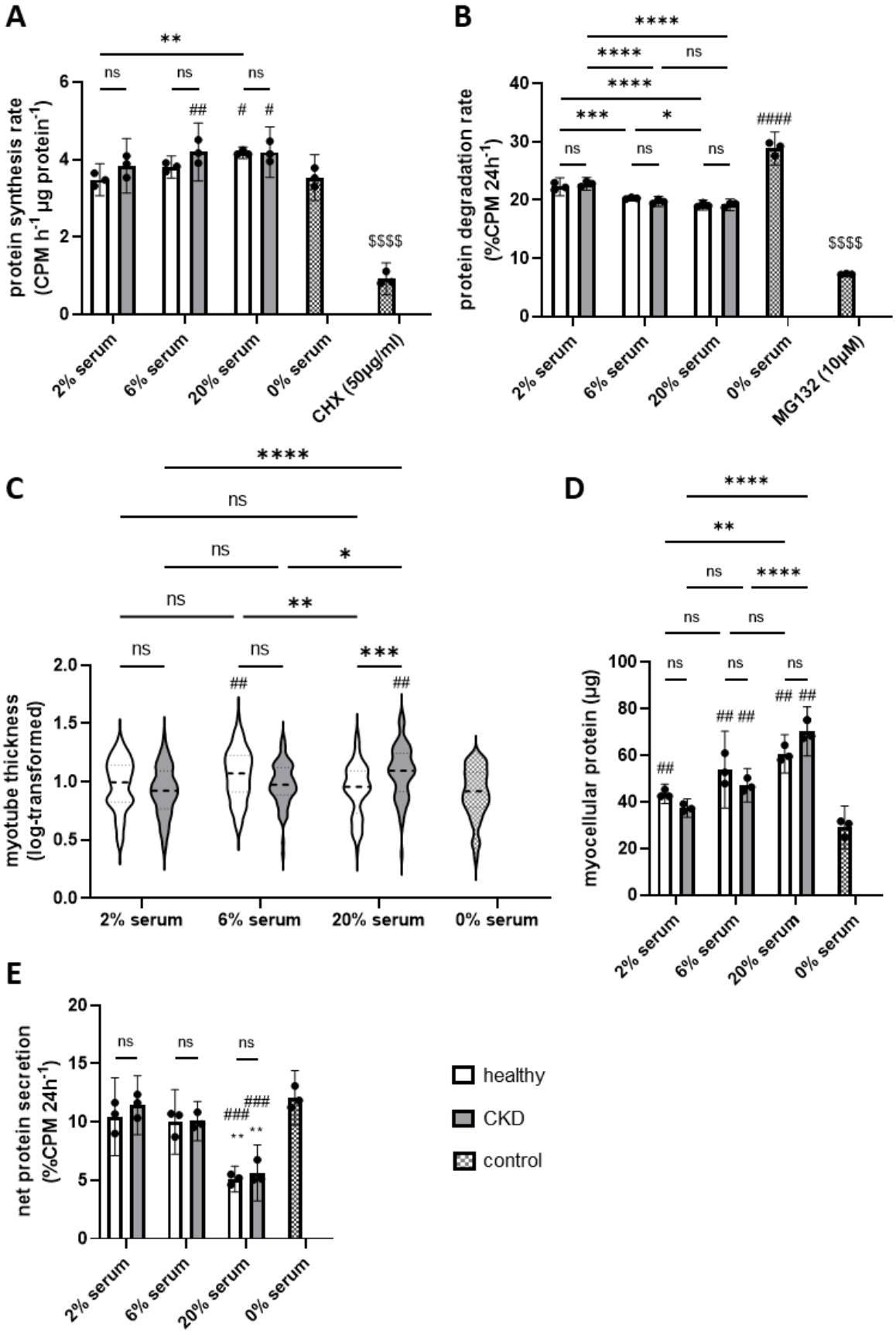


Figure 4.8 Initial characterisation of protein metabolism in human primary myotubes. ***A)*** *Protein synthesis rate as measured by incorporation of radiolabelled ^{3}H-tyrosine into human primary myotubes at standard conditions (control) and after treatment with cortisol for 24h.* ***B)*** *Protein degradation rate as measured by release of ^{3}H-tyrosine from pre-labelled intracellular proteins of human primary myotubes at standard conditions (control) and with cortisol treatment for 24h. Synthesis and degradation experiments performed in triplicate with cultures from one single primary human muscle cell lineage. Analysis by one-way ANOVA with Dunnett's multiple comparisons test.* ***C)*** *Protein synthesis rate and* ***D)*** *protein degradation rate of human myotubes exposed to 5% human serum from individual patients with non-alcoholic fatty liver disease (NAFLD) and matched control subjects (n=4 per group) for 24h. The synthesis and degradation experiment each used muscle cell cultures from one non-identical muscle tissue donor. Comparisons by unpaired t-test; ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001.*

protein synthesis as expected. Furthermore, protein synthesis rate increases significantly with rising concentration of human serum, regardless of CKD or healthy control condition. The results for protein degradation rate mirror the findings for protein synthesis rate. No differences in protein degradation are detectable between CKD serum and healthy control serum at concentrations of 2%, 6% or 20% (mean difference -0.48 CPM%/24h, 0.55 CPM%/24h and -0.02 CPM%/24h at 2%, 6% and 20% serum respectively, p>0.05 for all comparisons, Figure 4.9B). The proteosome inhibitor MG132 successfully suppresses protein degradation rate in the cultured muscle cells. Analogous to protein synthesis, protein degradation rate decreases significantly with increasing concentration of human serum, regardless of CKD or healthy control condition.

Figure 4.9 Effects of human serum from patients with CKD or healthy volunteers on protein metabolism in human primary myotubes.

[figure on following page] Human myotube cultures were exposed to pooled human serum from patients with CKD (n=6) or matched control subjects (n=5) for 24h. Control conditions include serum-free cell culture media (0% serum), the ribosome inhibitor cycloheximide (CHX) and the proteosome inhibitor MG132. ***A)*** *Protein synthesis rate for the outlined experiment.* ***B)*** *Protein degradation rate.* ***C)*** *Myotube thickness; data based on blinded analysis of phase-contrast light microscopy images using ImageJ software, taking 25 measurements per cell culture well for 3 cell culture wells per experimental condition. Thickness measurements log-transformed for analysis; thick lines indicate median and dotted lines indicate interquartile range.* ***D)*** *Cellular protein content of myotubes.* ***E)*** *Secretion rate of intracellular protein into culture media. Experiment performed in triplicate with cultures from one primary human muscle cell lineage. Analysis by two-way ANOVA with Tukey's multiple comparisons test; ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 for comparisons among human serum conditions; # p<0.05, ## p<0.01, ### p<0.001, #### p<0.0001 for comparisons of human serum conditions vs serum-free control condition; $$$$ p<0.0001 for comparison of any condition vs CHX or MG132.*


A
protein synthesis rate (CPM h-1 µg protein-1)
2% serum
6% serum
20% serum
0% serum
CHX (50µg/ml)
B
protein degradation rate (%CPM 24h-1)
MG132 (10µM)
C
myotube thickness (log-transformed)
D
myocellular protein (µg)
E
net protein secretion (%CPM 24h-1)
healthy
CKD
control

Myotube thickness and total myocellular protein content after 24-hour stimulation with CKD or healthy serum were assessed as complementary measures to protein synthesis and degradation rate. There is a trend for smaller myotube thickness with CKD serum compared to healthy serum at concentrations of 2% and 6%, but this does not reach statistical significance and the trend goes into the opposite direction at 20% serum concentration (difference in geometric means -1.08µm, -1.95µm and +3.65µm at 2%, 6% and 20% serum respectively, $p>0.05$ for all comparisons, Figure 4.9C). Corresponding with earlier results, myotube thickness is generally bigger following exposure to higher serum concentrations, although the condition of healthy serum at 20% is an outlier to this pattern. Results for total myocellular protein quantification correspond closely with findings from myotube thickness measurements. While there is no significant difference in protein content between CKD serum and healthy serum conditions, protein content is slightly reduced with CKD serum at 2% and 6% and slightly increased with CKD serum at 20% (mean difference -6.09µg, -6.74µg and +9.58µg at 2%, 6% and 20% serum respectively, $p>0.05$ for all comparisons, Figure 4.9D). Again, myocellular protein content is significantly greater with higher serum concentrations, independent of serum type.

Secretion of intact protein into the media was examined as a potential confounding variable. This measure is similar across tested conditions, except for a marked and equal suppression of protein secretion with 20% CKD or healthy serum treatment (Figure 4.9E). As the final experiment to corroborate the observed results, mRNA expression of anabolic and catabolic regulators was determined following 24h stimulation with 20% CKD or healthy control serum (Figure 4.10). Expression of catabolic markers (Foxo1, Fbxo32, Trim63), anti-anabolic markers

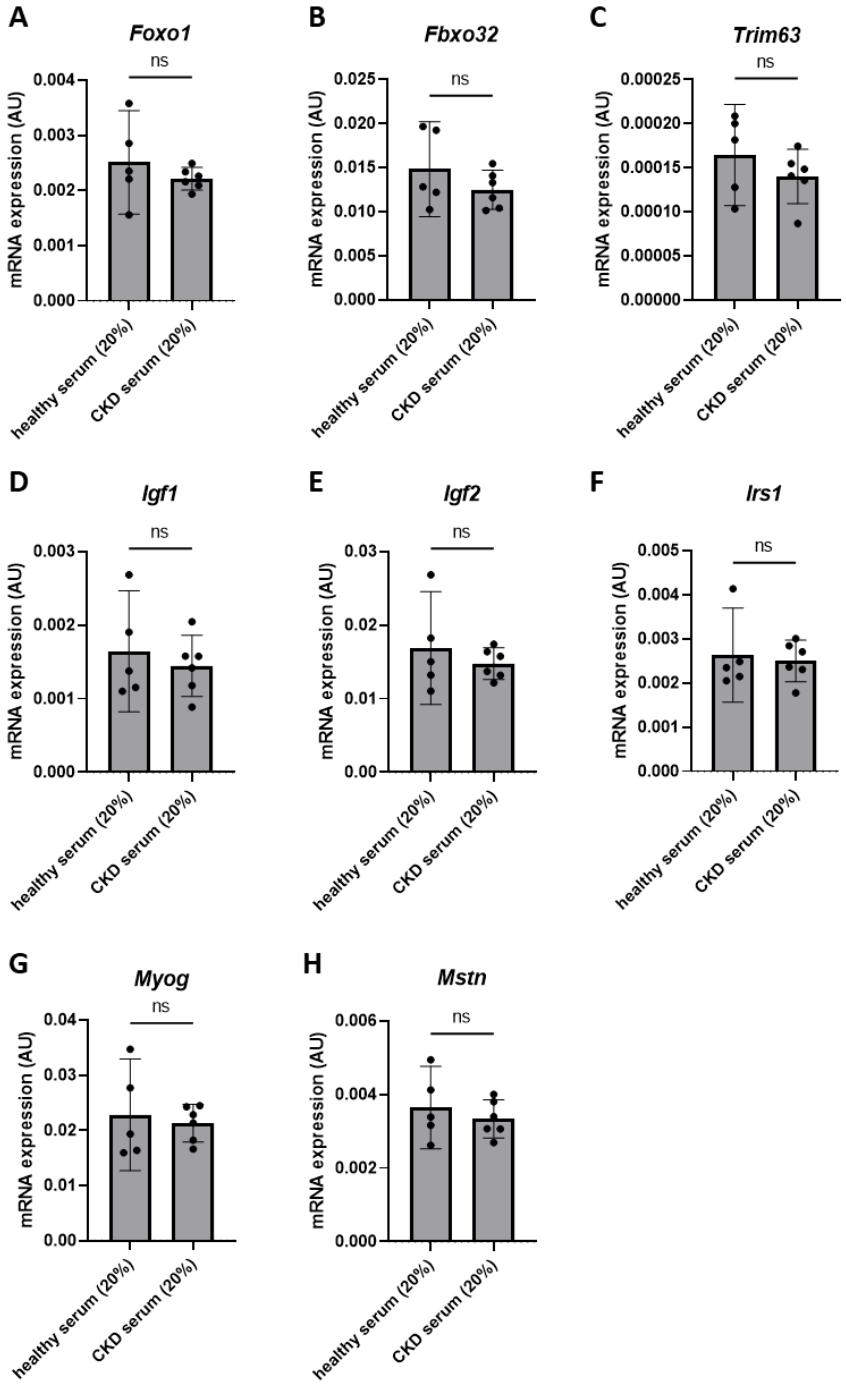


Figure 4.10 Effects of human serum from patients with CKD or healthy volunteers on mRNA expression of anabolic and catabolic genes in human primary myotubes.

mRNA expression of catabolic genes Foxo1, Fbxo32, Trim63 ***(A-C)****, anabolic genes Igf1, Igf2, Irs1* ***(D-F)****, myogenesis gene MyoG* ***(G)*** *and anti-anabolic gene myostatin* ***(H)*** *in human myotubes after treatment with 20% human serum from individual healthy donors (n=5) or patients with CKD (n=6) for 24h. All muscle cell cultures for this experiment had the same lineage derived from one muscle tissue donor. Comparison by two-tailed unpaired t-test; ns p>0.05.*

(myostatin), anabolic markers (Igf1, Igf2, Irs1) or differentiation markers (MyoG) are unchanged in the tested experimental conditions.

In summary, exploration of the effects of human serum from donors with CKD or healthy controls on protein homeostasis in primary human myotube cultures does not reveal any significant differential changes. The results are consistent across a range of assays including protein synthesis rate, protein degradation rate, myotube thickness, myocellular protein content and expression of metabolic regulators. Regardless of serum type, muscle cells respond to rising serum concentration with increased anabolic activity and decreased catabolic activity, highlighting the dynamic range of protein metabolism in this cell culture model.

4.3 Discussion

The aim for this research component was characterising the regulation of 11βHSD1 and its functional impact on myocellular protein metabolism under CKD-associated atrophic conditions in human primary muscle culture. The implemented cell culture protocol was successful in generating an experimental system of human myotubes that share differentiation markers with human skeletal muscle tissue and exhibit dynamic regulation of 11βHSD1 activity as well as protein metabolism. The inflammatory cytokine TNFα potently upregulated 11βHSD1 mRNA expression, enzymatic activity and downstream glucocorticoid signalling. Exposure of human myotubes to serum from humans with and without CKD did not elicit differences in 11βHSD1 enzymatic activity. Moreover, acidification of cell culture media had only a marginal effect on 11βHSD1 enzymatic activity in human myotubes. An exploratory experiment trialled a novel in vitro model of CKD serum-induced protein loss in human myotubes. However, the available results do not show robust differences for CKD serum compared to control serum in their effects on myocellular protein metabolism. In conclusion, the inflammatory cytokine TNFα emerges as the predominant regulator of 11βHSD1 activity in human muscle cell cultures among the examined conditions.

4.3.1 Characterisation of an experimental system based on human myotubes

All in vitro experiments were conducted in the experimental system of primary human myotubes. Previous research using in vitro models to study mechanisms of muscle wasting in conditions of kidney failure relied almost exclusively on muscle cell lines derived from mice or rats.[100, 377] Species differences present obvious limitations when it comes to translating

these scientific findings to human disease.[406, 407] Using human cells for in vitro models offers greater fidelity to human physiology and hence greater applicability to human disease. However, human muscle cell culture has inherent technical challenges.[408, 409] The precursor myoblasts are typically derived from fresh human skeletal muscle biopsies and undergo a multi-stage culture protocol over 20-50 days to yield non-proliferating differentiated myotubes. Through collaboration with Prof Simon Jones and his study on Muscle and Fat Cross Talk (IRAS ID 206880), it has been possible to access sufficient fresh skeletal muscle biopsy samples to conduct all in vitro experiments in primary human myotubes. The muscle tissue donors were men and women with osteoarthritis undergoing elective joint replacement surgery. This cohort shares important demographic characteristics with the CKD population, although muscle tissue donors typically did not have known renal impairment. In this sense, it has been possible to investigate the effects of circulating factors associated with CKD in a unique experimental system – an experimental system of human skeletal muscle tissue that is naïve to any embedded CKD-dependent phenotype yet derived from human tissue donors with similar demographic and metabolic risk profile as the population with CKD.

The differentiation profile of cultured human primary myotubes resembled human skeletal muscle tissue. This was confirmed through characterising the dynamic expression profile of progenitor and differentiation markers during the final differentiation phase of the cell culture protocol. The data show downregulation of progenitor markers Myf5, Myf6 and MyoD, with transient upregulation of myogenin. This is consistent with the recognised pathway for transition of myoblasts to myotubes.[407, 412] Microscopy furthermore confirm a change in morphology from fusiform to tubular, and immunofluorescence staining for the skeletal

muscle cell marker desmin confirmed that cultured myotubes indeed expressed this differentiation marker. Besides the verifications described here, the employed protocol has previously undergone extensive validation.[353, 416] Therefore, the experimental system can be regarded with a high degree of confidence as a model of human skeletal muscle tissue.

Differentiated human myotubes in vitro express 11βHSD1 and possess biological activity to convert inactive cortisone to active cortisone. The data reiterate several important properties of 11βHSD1 in cultured myotubes. First, differentiated myotubes have substantially higher 11βHSD1 enzymatic activity than proliferating myoblasts, which corresponds with previous studies.[326] Second, TNFα potently upregulates 11βHSD1 expression and 11βHSD1 biological activity in human myotubes. This is again consistent with extensive evidence in the literature, based on murine in vivo and human biopsy studies.[263, 351] The inflammatory cytokines TNFα and IL1B have the capacity to increase gene expression of 11βHSD1 via the proximal HSD11B1 gene promoter, acting via NF-κB signalling.[191, 263] Third, 11βHSD1 in human myotubes acts predominantly as an oxoreductase, i.e. converting cortisol to cortisone. This is suggested by the fact that conversion rate of cortisone to cortisone does not plateau up to 80% substrate conversion, indicating little reverse catalysis is taking place. Fourth, 11βHSD1 is the principal agent for converting cortisone to cortisol in human myotubes, as biological conversion rate is markedly altered by the 11βHSD inhibitor glycyrrhetinic acid and TNFα-induced upregulation of 11βHSD1 expression. This is further corroborated by observations from the 11βHSD1-KO animals. Finally, 11βHSD1 enzymatic activity determines the capacity of cortisone to activate downstream glucocorticoid signalling, as measured through expression of the glucocorticoid-responsive gene Gilz. Taken together, these findings confirm that primary

human myotube cultures possess biological 11βHSD1 activity with capacity for dynamic regulation. Human myotube cultures therefore represent an appropriate experimental system to investigate how CKD-associated circulating factors may influence 11βHSD1 function in skeletal muscle.

4.3.2 11βHSD1 activity in human myotubes does not differ following stimulation with serum from people with or without CKD

Human primary myotubes from donors without kidney disease were used in an experiment to compare the effects of human serum from donors with and without CKD on 11βHSD1 function. This research approach was chosen to evaluate the effect of CKD-related circulating factors in an unselected fashion, testing known and unknown potential mediators together. Similar strategies have been effective in multiple previous studies, although no reports were found in the literature for the specific experimental set-up of treating human myotubes with serum from humans with and without CKD.[324, 388, 395, 411, 417] Going against the original hypothesis, there was no evidence that serum from patients with CKD increased 11βHSD1 expression or function in comparison to serum from healthy volunteers in human myotubes. The following discussion will debate the experiment and its results in finer detail to consider whether the null hypothesis should be accepted. The discussion will explore pro-inflammatory properties of CKD serum as a potential determinant of 11βHSD1 function, inherent traits of the utilised human myotube cultures, adequacy of the selected serum samples and technical aspects of the experimental protocol.

Signalling via pro-inflammatory cytokine pathways could have offered a plausible mechanistic basis for the hypothesis of CKD serum-induced upregulation of 11βHSD1 in human myotubes. This study and others have shown that inflammatory cytokines TNFα or Il1β upregulate 11βHSD1 in muscle, and that pro-inflammatory cytokines circulate at higher concentrations in CKD than in health.[55, 263] Furthermore, serum C-reactive protein correlates independently with surrogate markers of systemic 11βHSD1 activity in CKD.[320] For the serum samples used in this experiment, it was confirmed that CRP levels were higher for serum donors with CKD than for healthy controls. Additionally, myotubes had verified capacity to respond to inflammatory stimuli like TNFα with upregulation of 11βHSD1 or Il6. Despite these preconditions, stimulation of human myotubes with CKD serum elicited no greater inflammatory response in myotubes than control serum, as measured by expression of Il6 mRNA or cumulative secretion of Il6 protein. Hence, elevated measures of systemic inflammation in CKD serum did not translate into elevated pro-inflammatory responses in CKD serum-treated myotubes in this experiment.

The design of the experiment accounted for the possibility that traits inherent to primary myotube cultures may bias the findings. It is a recognised feature of primary myotube cultures that phenotypic traits of the muscle tissue donor can be passed down to derived myotubes as specific phenotypic attributes.[409] For example, primary muscle cultures derived from people with obesity may exhibit insulin resistance in vitro compared to primary muscle cultures from lean tissue donors.[416] Accordingly, it is conceivable that a specific primary muscle cell lineage may harbour traits that confer resistance to CKD serum-induced 11βHSD1 upregulation, thus masking this effect. To mitigate against this risk, the experiment was

repeated in myotube cultures originating from muscle biopsies of four distinct persons picked at random. Reviewing the respective replicates individually, it appears unlikely that a genuine CKD serum-related effect on 11βHSD1 function in myotube cultures was masked by specific characteristics inherent to myotube cultures. Beyond that, it could be argued that the donor pool of patients with osteoarthritis is biased. However, the osteoarthritis population shares important characteristics with the CKD population. These include an aging demographic profile, elevated metabolic risk factors and reduced physical activity levels, justifying the use of this donor pool for this experiment. Switching to a different donor pool for primary cultures or to an alternative cell culture system is likely to introduce further biases of equal or greater magnitude. For these reasons, the employed cell culture system provided an appropriate – even if imperfect – framework for testing the research hypothesis.

The characteristics of donors for serum samples to be used in the experiment were carefully considered. Serum donors with CKD and normal health had a big difference in kidney function, while being matched for age and gender. Higher CRP and higher HbA1c in CKD serum reflected inflammatory activation and impaired glycaemic control that are typical for the CKD population.[26, 55, 141] Hence, there was clear separation of serum donors by clinical disease status, as intended. It is possible that pathological changes in the used serum samples, originating from community-dwelling patients with moderate to severe renal impairment and unknown muscle phenotype, are less pronounced than would be expected for patients with end-stage renal failure and established sarcopenia.[55, 418] Whether serum from such patient groups has different effects on myotubes compared to the serum from pre-dialysis patients with CKD was not examined in this study and could form part of future research. Yet, the

impact of dialysis treatment itself on patients' health could introduce a potential confounder. It is reasonable to assert therefore that the examined serum samples were appropriate for addressing the research question (whether CKD-associated circulating factors influence 11βHSD1 function in myotubes), based on the serum donors' known clinical and demographic characteristics.

The experimental design for in vitro studies with human serum treatments was based on careful consideration of the literature. Two concentrations of human serum – 10% and 20% – were tested over 48 hours. Similar studies in the literature have used 5-20% healthy or uraemic human serum to treat muscle cells or primary human liver cells.[324, 417, 419, 420] These conditions elicited wide-ranging differences in cellular metabolism, gene expression and signalling cascades. Time-response curves from these studies indicated that differential responses in gene transcription take 24-48 hours to emerge.[324, 417, 420] For example, 5% uraemic serum stimulation over 24 hours induced upregulation of 11βHSD1 gene expression in human hepatocytes.[324] This corresponds with time-response studies for 11βHSD1 regulation in human skeletal muscle, showing transcriptional upregulation over 24 hours.[263] A 48-hour pre-treatment period was chosen for 11βHSD1 activity assays to allow for changes in protein translation and downstream metabolic responses. Furthermore, sustained myocellular responses were deemed more likely to have a biologically relevant impact on protein balance and atrophy. Therefore, previous optimisation studies and considerations of biological relevance provided a clear rationale for the concentration and duration of serum treatments in the in vitro experiments.

In summary, potential sources of bias in serum-treated myotube experiments were addressed through using primary human cells from a range of tissue donors and testing human serum samples from a range of donors with clear separation by kidney disease status. The dose and exposure time were based on previous optimisation studies and biological considerations to offer the greatest chances for detecting differences. Having taken these precautions to enhance the validity of the results, the experiment found no difference in 11βHSD1 function of cultured human myotubes upon stimulation with serum from donors with and without CKD. Ultimately, it will be important to interpret these results together with data from in vivo models of kidney disease and human patients with CKD to clarify the regulation of 11βHSD1 in kidney failure.

4.3.3 Acidosis effects on human myotubes in vitro

Acidosis effects on myotubes and 11βHSD1 regulation were investigated separately. Treatment of myotubes with uraemic serum does not replicate this aspect of renal impairment, as mixing serum with cell culture media can dilute or eliminate subtle metabolic derangements. Acidosis is known to contribute to muscle wasting via a mechanism that requires glucocorticoid signalling as a cofactor, but the role of 11βHSD1 has been unclear.[126, 256] Validation studies were carried out to demonstrate that pH in media could be stably maintained in the context of cell culture buffer systems. Media exposed to cells had slightly lower pH than media stored in blank wells in the tissue incubator after 48 hours. This is a plausible finding, reflecting metabolic conversion of glucose to lactate. Acidification of media did not induce gross cellular toxicity, as assessed by Trypan Blue staining. Tolerance of extracellular acidification to low physiological levels without undue toxicity is also validated in the literature.[421] In an

additional step to validate the acidosis condition, reduction in insulin-stimulated glucose disposal was demonstrated. This corresponds with the body of literature that acidosis reduces insulin sensitivity.[143, 421-423] Therefore, the data verify successful establishment of a sustained extracellular acidosis condition in human myotube cultures that induced measurable changes in cellular metabolism.

Treatment of human myotubes with acidified media produced a small reduction in 11βHSD1 activity. However, this observation could not be validated in follow-up experiments, calling into question the significance of this observation. A statistically significant reduction in 11βHSD1 enzymatic activity was found at pH7.1 and pH6.8 compared to pH7.4, with a relative reduction in cortisol production rate of 12% and 13% respectively. This represents an aggregate result of myotube cultures derived from four distinct tissue donors. Abundance of 11βHSD1 mRNA showed no correlating downregulation, pointing towards changes in post-translational processing, co-factor availability or catalytic efficiency as explanations for putative reduction in cortisol production rate. When a follow-up experiment aimed to examine the effects on downstream glucocorticoid signalling through measuring cortisone-mediated Gilz expression, the data were inconsistent with reduced 11βHSD1 activity, showing slightly higher Gilz expression in the acidosis condition instead. It is unlikely that acidosis interfered directly with glucocorticoid receptor signalling, as no pH-dependent effects were seen with cortisol-induced Gilz upregulation. Two scenarios may account for these diverging results. On the one hand, the results could be influenced by inherent genetic and epigenetic traits of myotubes, as primary cultures from different tissue donors were used in these experiments. On the other hand, the previously observed reduction in 11βHSD1 activity could be an artefact, relating to

changes in myocellular protein content which is used a measure of tissue mass for normalising enzymatic rate measurements. Regardless of the specific reason, the relatively small magnitude and conflicting direction for observed effects call into question whether alterations to 11βHSD1 function in acidosis conditions are real and biologically relevant. Further information in this regard is presented by a study of human volunteers who experienced acute systemic acidosis, which found no change in the 11βHSD1 surrogate marker of urinary (THF+5αTHF)/THE ratio.[254] Based on these considerations, a significant shift in 11βHSD1 function in response to acidosis was deemed unlikely and further investigations on this question ceased.

4.3.4 Effects of serum from humans with and without CKD on protein metabolism in human myotubes

The original research objectives included investigation of 11βHSD1-dependent effects on myocellular protein metabolism under CKD-associated atrophic conditions in human primary muscle culture. The atrophic effects of glucocorticoids by themselves, in combination with 11βHSD1 or in combination with acidosis have already been investigated extensively in muscle cell culture systems.[222, 256, 326, 424] When it became apparent that CKD serum or acidosis do not exert major effects on 11βHSD1 function in myotubes, the line of investigation was revised. Efforts focused instead on establishing a novel in vitro model of CKD-related muscle atrophy by stimulating primary myotubes derived from humans without CKD with serum from humans with CKD. Similar experimental strategies have been successful in related settings. Li et al. demonstrated changes in gluconeogenesis and intracellular lipid accumulation in human primary hepatocytes following exposure to human uraemic serum.[324] Other studies with

uraemic serum have shown changes to energy metabolism and myotube atrophy in C2C12 murine muscle cell cultures.[388, 395] Protein metabolism has been characterised in some detail in C2C12 cells following stimulation with serum from aged humans and patients with liver disease, identifying changes in muscle protein synthesis and protein breakdown signalling respectively.[411, 417] However, reliance on a murine immortalised muscle cell line is a limitation with all of these studies. Establishing a uraemic serum-induced model of muscular atrophy and muscle protein loss in human myotubes should improve validity in terms of pathophysiology and evaluation of therapeutic strategies.

Prior to trialling CKD serum as an atrophic stimulus, it was important to verify the capacity of primary human myotubes for dynamic regulation of protein metabolism and the reliability of assays to detect changes in protein synthesis and degradation. Assays employed the amino acid tyrosine as a radiolabelled tracer of protein synthesis and degradation – a methodology which interferes minimally in endogenous metabolism and has a long history of producing reliable data in diverse biological systems.[95, 326, 425-427] When cortisol was tested as an agent with known anti-anabolic and catabolic properties, myotubes exhibited dose-dependent reductions in protein synthesis rate and dose-dependent increases in protein degradation rate. Furthermore, exposure of myotubes to serum from patients with non-alcoholic fatty liver disease increased protein degradation rate compared to serum from healthy individuals, but did not significantly alter protein synthesis rate. This pattern replicates previous observations obtained from C2C12 cells.[411] The cell culture system and protein turnover assays therefore appeared suitable for the specified research objective.

Exploratory experiments found no consistent pattern for changes in myocellular protein metabolism with CKD serum. To reduce the risk of false negative results, a range of serum concentrations were tested and serum-free or inhibitor conditions were included as experimental controls. Protein synthesis and degradation measurements did not show significant differences between pooled sera from patients with CKD and pooled sera from age-matched healthy volunteers. If anything, a non-significant trend suggested higher protein synthesis rate with CKD serum at lower concentrations. Validity of the results is supported by appropriate changes in protein turnover measurements in relation to serum concentration, regardless of serum type, and appropriate effects of control conditions. To further corroborate the findings from protein turnover measurements, myotube thickness and total myocellular protein content were quantified. Despite a trend for muscle cell atrophy with CKD serum at 6% serum concentration and less convincingly at 2% serum concentration, effect direction was reversed at 20% serum concentration. While no comparisons by serum type reached statistical significance, again there were statistically significant differences with serum concentration. Tested only at 20% serum concentration, mRNA expression of anabolic and catabolic genes did not differ for CKD and control serum conditions. To sum up these results, there was no reliable evidence for differential effects of serum from humans with and without CKD on myocellular protein metabolism within the scope of examined experimental conditions. Appropriate results for integrated control conditions support the validity of the experimental findings. Nevertheless, certain limitations with this experiment should be considered before generalising its findings.

The experiment was exploratory in nature. In order to strengthen the argument that uraemic human serum has no different effect on protein metabolism of human myotubes than healthy serum, a range of further systematic experiments are required. First, it will be necessary to repeat the experiment in primary myotubes originating from multiple tissue donors. So far, only one lineage of primary myotubes has been tested, making the results susceptible to bias from unique inherent genetic and epigenetic traits. This is a more stringent criteria than usual in cell culture experiments, which commonly are conducted only in a single immortalised lineage. Second, it will be beneficial to test serum from donors individually, rather than testing pooled serum samples. Samples were pooled for the experiment as they were limited in supply and it was important to ensure identical conditions for experimental replicates. Serum pooling in this setting is commonly described in the literature and has produced meaningful results.[324, 395] Ideally, stimulation with serum from individual donors would be preferable though. This would avoid the theoretical risk of negating the individual properties of a serum sample through the pooling process. Third, testing a greater number of different serum samples will give higher confidence in the results. Another way to address unwarranted experimental variability is to collect serum samples under controlled conditions. Factors such as time of day, time since last meal or environmental setting may exert a relevant impact through variation in hormone levels, metabolites or other mediators. Fourth, testing serum samples from donors with known muscle phenotype will be helpful for interpreting the result. Although key demographic and clinical features were known for the serum samples used in the experiment, it is not possible to tell from the available data whether donors' muscle phenotype may have impacted on or biased the results. Finally, differential effects of serum from people with and without CKD may be masked in the baseline condition. Possibly,

differences in myotube responses may only emerge upon introducing a secondary challenge, e.g. nutrient withdrawal or pro-inflammatory stimuli. Such phenomena are common for anabolic resistance, where introduction of a growth stimulus is necessary to evoke phenotypic differences that are masked at baseline.[4, 417, 428] All of these additional experiments will provide useful information about the capacity of uraemic serum to modulate protein metabolism in human myotubes in vitro. At the same time, adhering to all of these premises is a challenge in the face of finite availability of muscle tissue and serum samples, and their inherent biological variability. That challenge reduces the practicality of this approach as a model of CKD-related muscle cell atrophy.

The null hypothesis remains a possibility, that CKD serum has no different effect on primary human myotubes than healthy serum in vitro over a period of 24-48h, even after controlling for all the discussed sources of bias. Analytic techniques with broad unbiased analysis could provide evidence to strengthen the null hypothesis, e.g. RNA sequencing or mass spectrometry-based proteome or metabolome characterisation. Even if this specific null hypothesis were accepted, it need not contradict the recognised sarcopenia risk in CKD. Alternative explanations include that circulating factors act over longer timescales to effect change in muscle cells, that circulating factors act at earlier stages of muscle cell differentiation, that myopathic effects in CKD are caused by transient circulating factors, that muscle cells in vivo respond differently to circulating factors than muscle cell in vitro, or that circulating factors are of minor importance for sarcopenia in CKD. An interesting study to mention at this point was carried out by Baker et al. at University of Leicester.[428] Primary human muscle cell cultures derived from individuals with CKD had higher rates of protein

degradation and resistance to IGF-1 stimulation of protein synthesis compared to muscle cell cultures from healthy individuals. Besides proving the carry-over of phenotypic traits from tissue donor to primary culture, the findings propose imprinting of CKD-related atrophic stimuli in muscle progenitor cells.

In conclusion, an exploratory experiment could not readily achieve a model of myotube atrophy induced by human CKD serum in vitro. Further research is needed to assess the feasibility of this approach. This research may in itself advance knowledge about the pathophysiology of CKD-related sarcopenia, in addition to delivering a novel experimental model. However, the scope of this endeavour should not be underestimated, given the necessity to deal with biological variability and finite supply of primary human muscle tissue and human serum samples.

4.3.5 Limitations

A range of important limitations and additional considerations concerning the in vitro experiments have already been discussed. For completeness, several additional limitations with the experiments should be noted:

- Cell culture always occurs in an artificial environment that is an imperfect replication of in vivo conditions. Lack of three-dimensional architecture, neuronal innervation or physical forces are but a few examples of many differences in environmental conditions that may alter the phenotype of cultures myotubes, thereby limiting generalisability of results.

- Experiments were conducted with awareness that genetic and epigenetic traits inherent in primary myotubes may influence the results. To address this risk, principal experiments were repeated in myotubes originating from different donors. However, there were no further efforts to actively control for myotube donor traits, for example through a systematic exploration whether specific donor traits influence observations in a specific direction. Given the additional resources and effort required for such work, it was deemed outside the scope of this study.

- The precise collection conditions for serum samples used in the experiments are not known. Many biochemical differences in serum from people with and without CKD are profound and relatively stable over time. Nevertheless, circulating hormones, nutrients, metabolites and other factors that regulate 11βHSD1 and myocellular protein metabolism vary according to time of day, feeding status, preceding activity and environmental setting. This represents a potential source of unwarranted variation in the experimental results, which should be rectified in future studies with a similar approach.

- It was not possible to confirm any difference in phenotype between CKD serum-treated myotubes and control serum-treated myotubes. Without such a positive confirmation, technical insufficiencies relating to cell culture, serum samples or experimental conduct cannot be completely excluded. The observation that serum from patients with liver disease induced a detectable increase in protein degradation is partly reassuring in this regard. To address questions about the adequacy of the experimental parameters, future research will involve a broad unbiased analysis of myotubes by

RNAseq following stimulation with a greater number of individual serum samples from donors with and without CKD. Data from this experiment is pending.

- Characterisation of the serum samples used as experimental conditions relied largely on donors' demographic and clinical characteristics. Further detailed analysis of serum constituents such as inflammatory cytokines, hormone levels and growth factors would be helpful for the interpretation of the results. Still, such additional analysis will not alter the outcome of the conducted experiments, as their design purposefully allowed for the effects of unknown factors.

4.3.6 Summary

In vitro experiments using primary human muscle cells did not reveal differences in 11βHSD1 function following exposure to serum from patients with or without CKD. Acidification of cell culture media marginally reduced 11βHSD1 in human primary muscle cells, but the small effect size and inconsistent follow-up data weaken the reliability of this result. Lack of clear changes in 11βHSD1 function with these tested conditions occurred despite carefully characterising the suitability of the experimental system and controlling for potential sources of bias. Data confirmed that the inflammatory cytokine TNFα potently upregulates 11βHSD1 expression, activity and downstream glucocorticoid signalling in human primary muscle cells. As with the in vitro experiment, no significant change in muscle 11βHSD1 function ex vivo was observed in the mouse model of renal impairment. Investigation of 11βHSD1 activity in muscle tissue from people with CKD will hold the potential to deliver concluding validation or refusal of these preclinical findings.

Chapter V – Studies in humans to compare glucocorticoid metabolism in health and in CKD and examine correlations with sarcopenia risk

5.1 Introduction

5.1.1 Rationale for experimental approach

This third results chapter discusses observational data on systemic and muscle-specific measures of glucocorticoid metabolism in humans with a range of kidney function. The previous chapters on 11βHSD1 in preclinical models of CKD provide valuable insights into its regulation in skeletal muscle and its role in the pathophysiology of sarcopenia. Ultimately however, it is essential to corroborate and validate knowledge from preclinical models directly in human disease to eliminate potential fallacies. Only studies in humans can fully encapsulate all aspects of physiology and pathophysiology for skeletal muscle in CKD, including influences from behavioural factors, comorbidities and treatments.

The primary goal for the following clinical investigations is to examine the role of 11βHSD1 in skeletal muscle for muscle mass and function in human CKD. Observational study methodologies will be employed to gather evidence for or against the primary hypothesis that overactivity of 11βHSD1 in skeletal muscle contributes to sarcopenia in CKD. As such, the generated data may confirm or exclude associations between 11βHSD1 activity and CKD or sarcopenia risk, although it cannot conclusively determine causal relationships. An observational approach is chosen instead of an interventional design, because prevailing uncertainty on 11βHSD1 as a risk factor or therapeutic target for sarcopenia in CKD make it difficult to justify the expense and potential harms associated with a clinical trial at this point in time. To fill this gap, complementary interventional and mechanistic studies in preclinical models have been reported in the previous chapters.

Observational data from two sources will be analysed in the following chapter. The first dataset is obtained from the Renal Impairment in Secondary Care (RIISC) study, a cohort study recruiting participants with CKD stage 3-5 between 2010 and 2015.[320, 356] The RIISC study had been established as an exploratory study to identify risk factors for CKD progression, morbidity and mortality. The large sample size, diverse representation of patients with CKD and available data on urinary steroid metabolome allow exploration of associations between glucocorticoid metabolism and sarcopenia risk factors with statistical adjustment for potential confounders. However, direct measures of 11βHSD1 or muscle phenotype are not available in this dataset, restricting inquiries to analysis of surrogate markers.

The ratio of urinary cortisol and cortisone metabolites (THF+5αTHF)/THE is used to gauge peripheral activation of circulating glucocorticoids in the analysis of RIISC study data. This measure is sensitive to systemic changes in 11βHSD1 activity in humans.[372, 429] At the same time, the ratio of active and inactive glucocorticoids is also influenced by the rate of glucocorticoid inactivation catalysed by 11βHSD2. CKD is associated with reduced 11bHSD2 expression in the kidney, the main site of 11βHSD2 activity.[301] The urinary (THF+5αTHF)/THE ratio therefore has inherent limitations as a surrogate marker, as it lacks specificity to identify tissue-specific changes in glucocorticoid metabolism or discriminate between 11βHSD1 and 11βHSD2 changes.

To address this limitation, data from a second study is evaluated, the Steroid Metabolism and Muscle Loss in Chronic Kidney Disease (SMK) study.(ISRCTN15497320) This is an original and purpose-specific case-control study involving participants with CKD stage 4-5 and healthy

volunteers recruited between 2022 and 2023. Even though the sample size is smaller, muscle phenotype and muscle 11βHSD1 activity are directly measured in this study, facilitating more accurate comparisons. Together, the two datasets from the RIISC and SMK studies provide a comprehensive picture on glucocorticoid metabolism in CKD and its role for muscle mass and function.

5.1.2 Chapter aim and objectives

The following analysis of two observational datasets will explore associations between systemic measures of glucocorticoid metabolism, muscle-specific 11βHSD1 activity, kidney function and sarcopenia risk.

Aim

3. Assess variation in muscle 11βHSD1 activity in relation to kidney function, and explore 11βHSD1 activity as a risk factor for sarcopenia

Objectives

a) Analyse correlations between surrogate markers of 11βHSD1 activity and risk factors for sarcopenia using data from a previous CKD cohort study (RIISC study)
b) Compare and contrast participants' characteristics and muscle phenotype depending on CKD status in an original study on glucocorticoid metabolism (SMK study)
c) Compare 11βHSD1 activity in skeletal muscle biopsies from people with and without CKD (SMK study)

d) Characterise differences in systemic glucocorticoid balance between people with and with CKD (SMK study)

e) Explore variation in muscle 11βHSD1 activity as a predictor of sarcopenia risk (SMK study)

5.2 Results

5.2.1 Urine (THF+5αTHF)/THE ratio as a surrogate for systemic 11βHSD1 activity weakly associates with restrictions in activities of daily living in the Renal Impairment in Secondary Care (RIISC) study cohort

The Renal Impairment in Secondary Care cohort study has previously characterised a sample of the CKD population, including data on glucocorticoid steroid profiles in urine. This study and others have shown that peripheral metabolism by 11βHSD enzymes shifts in favour of glucocorticoid activation in CKD, as measured by the ratio of active and inactive glucocorticoid metabolites (THF+5αTHF)/THE in urine.[300, 301, 320] The existing RIISC cohort data set therefore provides an opportunity to explore links between 11βHSD1 metabolism and skeletal muscle in human CKD. Building on the existing RIISC cohort dataset, additional analyses were performed with the aim to test associations between urinary (THF+5αTHF)/THE ratio as a surrogate for systemic 11βHSD1 activity and risk factors for sarcopenia. The demographic and clinical characteristics of the study cohort have previously been reported and are repeated here for ease of reference (Table 5.1).[320]

CKD patients' self-reported capability to perform usual activities of daily living was examined in relation to urinary (THF+5αTHF)/THE ratio. This variable is used as a proxy measure of skeletal muscle function, as the RIISC cohort study did not gather direct measurements for skeletal muscle mass, strength or function. Individuals with severe restriction in usual daily activities have elevated urinary (THF+5αTHF)/THE ratios compared to people experiencing no or only some restrictions in usual activities ($p=0.007$ and $p=0.040$ respectively, Figure 5.1A). To

explore this finding further and investigate potential confounding variables, multivariable logistic regression analysis was performed. As the number of cases in the severe restriction category was low, the categories of some restriction and severe restriction in daily

Table 5.1 Participant characteristics for sample population from RIISC cohort

Variable	Value	
n	325	
Age (years)	61.4	(49.9-75.2)
Female	126	(39%)
Ethnicity		
White	227	(70%)
South Asian	62	(19%)
Black	33	(10%)
Other	3	(1%)
Aetiology of renal disease		
Ischaemic/hypertensive	67	(21%)
Glomerulonephritis	69	(21%)
Polycystic	31	(10%)
Diabetic	31	(10%)
Obstructive/reflux	24	(7%)
Interstitial disease	18	(6%)
Not known and other	85	(26%)
Renal function markers		
Serum creatinine (µmol/l)	199	(145-281)
$eGFR_{creat}$ (mL/min/1.73 m2)	27	(17-41.1)
Serum cystatin C (mg/l)	2.31	(1.73-3.3)
$eGFR_{cysC}$ (mL/min/1.73 m2)	24.3	(15.1-36.5)
Urine ACR (mg/mmol)	41.4	(6.1-125)
Diabetes mellitus	121	(37%)
C-reactive protein (mcg/mL)	2.82	(1.32-6.82)
Urine (THF+5αTHF)/THE ratio	1.26	(0.89-1.82)

Continuous variables are reported as median (interquartile range) and categorical variables as frequency (percentage).
ACR, albumin-creatinine ratio; $eGFR_{creat}$, estimated glomerular filtration rate based on serum creatinine; $eGFR_{cysC}$, estimated glomerular filtration rate based on serum cystatin C.

activities were combined to avoid statistical issues related to model overfit. In the binary logistic regression model for no versus any restriction in daily living as outcome variable, log-transformed urinary (THF+5αTHF)/THE has a borderline significance value of p=0.052 when it is included as the only independent variable (odds ratio 1.528, 95% C.I. 0.995-2.344, Table 5.2). The expanded multivariable logistic regression model indicates that gender, age, C-

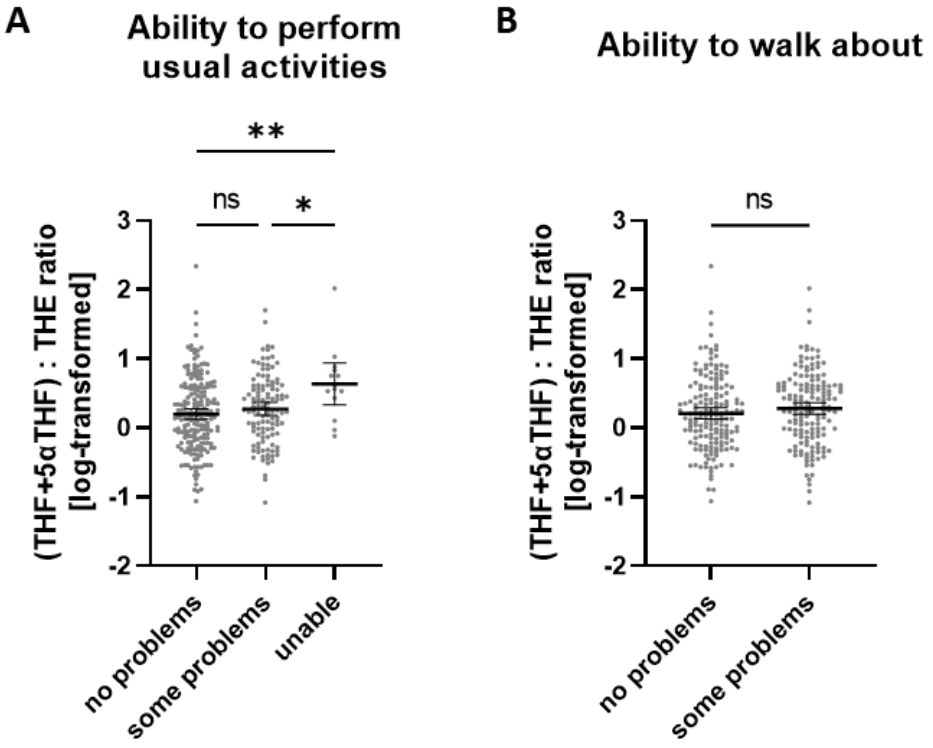


Figure 5.1 Association of urine (THF+5αTHF)/THE ratio with ability to perform activities of daily living among people with CKD.

A) *Urine (THF+5αTHF)/THE ratio for people with CKD who report no problems with performing usual activities, have some problems with performing usual activities or are unable to perform usual activities.* ***B)*** *Urine (THF+5αTHF)/THE ratio for people with CKD who report no problems in walking about or have some problems in walking about. Data from RIISC cohort, n=325. Urine (THF+5αTHF)/THE ratio was transformed with the natural log function for analysis. Analysis using one-way ANOVA with Tukey's multiple comparison test or unpaired t-test; ns p>0.05, * p<0.05, ** p<0.01.*

reactive protein as an inflammation marker and diabetes diagnosis independently predict restrictions in daily activities, but not urinary (THF+5αTHF)/THE ratio or kidney function quantified as eGFR (Table 5.2). An additional analysis of data on self-reported mobility problems shows no difference in urinary (THF+5αTHF)/THE ratios between individuals with no mobility restrictions and those with some restriction in mobility (Figure 5.1). As far as can be assessed within the RIISC cohort dataset, evidence for associations between urinary (THF+5αTHF)/THE ratio and self-reported problems in daily living or mobility is weak or absent.

Table 5.2 Binary logistic regression model for at least some problems to perform usual activities

			95% C.I. for OR		
Model	**Variable**	**OR**	**Lower**	**Upper**	**p**
univariable	(THF+5αTHF)/THE*	1.528	0.995	2.344	0.052
multivariable	(THF+5αTHF)/THE*	1.251	0.777	2.012	0.357
	Female gender	1.825	1.084	3.073	0.024
	Age (year)	1.038	1.019	1.057	<0.001
	eGFR (ml/min/1.73m^2)	1.005	0.992	1.018	0.435
	CRP – low tertile$^{\$}$	ref.			
	CRP – middle tertile	1.305	0.700	2.432	0.402
	CRP – high tertile	2.664	1.421	4.991	0.002
	Diabetes diagnosis	2.409	1.455	3.987	0.001

Data from RIISC cohort, n=325. The categories "some problems to perform usual activities" and "unable to perform usual activities" were treated as a pooled outcome variable and contrasted with "no problems to perform usual activities" for this regression analysis to avoid model overfit.

** ratio of (THF+5αTHF) : THE in urine, included as log-transformed variable.*

$^{\$}$ C-reactive protein, included as stratified variable to comply with linearity assumption (limits: low tertile ≤1.828µg/mL – reference for comparison, middle tertile 1.828µg/mL – 4.509µg/mL and high tertile >4.509µg/mL)

OR, odds ratio; C.I., confidence interval.

5.2.2 Urine (THF+5αTHE)/THE ratio is weakly associated with a surrogate for skeletal muscle mass derived from serum cystatin C and creatinine in the RIISC study cohort

To investigate putative links between urinary (THF+5αTHF)/THE ratio and skeletal muscle mass in the RIISC cohort, a surrogate marker derived from serum cystatin C and serum creatinine was used. While no direct measurements of muscle mass were taken in the RIISC study, the ratio of eGFR based on serum cystatin C (eGFR(cysC)) divided by eGFR based on serum creatinine (eGFR(creat)) has previously been shown to correlate with lean tissue mass.[430, 431] To begin with, the eGFR(cysC)/eGFR(creat) ratio was assessed in relation to self-reported problems in daily living or mobility. This was done to provide a degree of internal validation for this variable as a surrogate marker of muscle mass. Indeed, eGFR(cysC)/eGFR(creat) ratio declines successively with increasing restrictions in usual activities (Figure 5.2A). Furthermore, eGFR(cysC)/eGFR(creat) ratio is significantly lower in individuals who reported restriction in mobility (Figure 5.2B).

Analysis then proceeded to investigating the correlation between eGFR(cysC)/eGFR(creat) ratio as a marker of muscle mass and urinary (THF+5αTHF)/THE ratio as a marker of 11βHSD1 activity. Urinary (THF+5αTHF)/THE ratio is significantly correlated with eGFR(cysC)/eGFR(creat) ratio (Pearson r = -0.119, p=0.038, Figure 5.2C). However, after adjusting for gender, age, diabetes, C-reactive protein and severity of renal impairment based on a serum cystatin C threshold value as potential confounders in a multivariable linear regression model, urinary (THF+5αTHF)/THE ratio is no longer independently correlated with eGFR(cysC)/eGFR(creat) ratio (Table 5.3). As far as it is possible to assess within the RIISC cohort dataset, the association between systemic 11βHSD1 activity inferred from urinary (THF+5αTHF)/THE ratio and muscle

mass inferred from eGFR(cysC)/eGFR(creat) ratio is relatively weak and does not persist after adjustment for potential confounders.

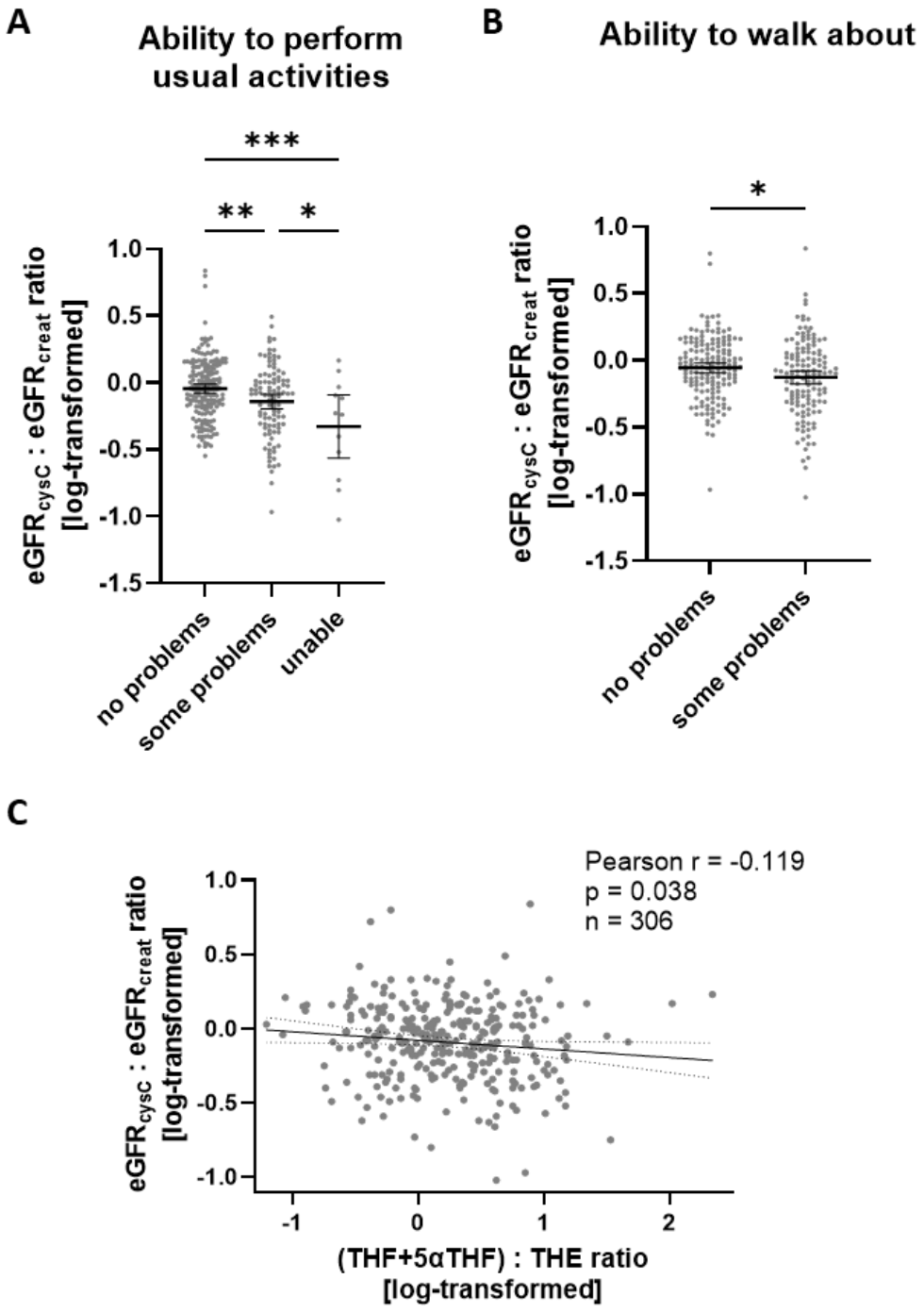


Figure 5.2 The ratio of cystatin C-derived eGFR to creatinine-derived eGFR and its association with activities of daily living and urine (THF+5αTHF)/THE ratio among people with CKD.

[figure on previous page] ***A)*** *The ratio of serum cystatin C-derived eGFR ($eGFR_{cysC}$) to serum creatinine-derived eGFR ($eGFR_{creat}$) for people with CKD who report no problems with performing usual activities, have some problems with performing usual activities or are unable to perform usual activities. Analysis by one-way ANOVA with Tukey's multiple comparisons test.* ***B)*** *$eGFR_{cysC}$: $eGFR_{creat}$ ratio for people with CKD who report no problems in walking about or have some problems in walking about. Comparison by unpaired t-test.* ***C)*** *Correlation between $eGFR_{cysC}$: $eGFR_{creat}$ ratio and urine (THF+5αTHF)/THE ratio. Analysis using Pearson correlation test, solid line indicates simple linear regression fit and dotted curves indicate 95% confidence interval for regression line. Data from RIISC cohort, n=306. Urine (THF+5αTHF)/THE ratio and $eGFR_{cysC}$: $eGFR_{creat}$ ratio were transformed with the natural log function for analysis. * p<0.05, ** p<0.01, *** p<0.001.*

Table 5.3 Linear regression model for log-transformed $eGFR_{cysC}$: $eGFR_{creat}$

			95% C.I. for B				
Model	**Variable**	**B**	**lower**	**upper**	**β**	**t**	**p**
univariable	(THF+5αTHF)/THE[1]	-0.058	-0.113	-0.003	-0.118	-2.079	0.038
multivariable	(THF+5αTHF)/THE[1]	-0.041	-0.097	0.014	-0.085	-1.474	0.141
	Female gender	0.045	-0.014	0.105	0.085	1.507	0.133
	Age (year)	4.79×10^{-4}	-1.38×10^{-3}	2.34×10^{-3}	0.031	0.507	0.613
	CysC >2.31mg/l[2]	-0.086	-0.149	-0.023	-0.166	-2.697	0.007
	Diabetes diagnosis	-0.101	-0.163	-0.04	-0.187	-3.247	0.001
	CRP (µg/ml)	1.26×10^{-3}	-8.64×10^{-4}	3.39×10^{-3}	0.068	1.168	0.244

Adjusted R^2=0.011 for univariable model and R^2=0.060 for multivariable model. Data from RIISC cohort, n=306.

[1] ratio of (THF+5αTHF) : THE in urine, included as log-transformed variable.

[2] serum cystatin C, included as binary variable above median cystatin C value of 2.31mg/l in study population.

CRP, C-reactive protein; C.I., confidence interval.

5.2.3 Baseline participant characteristics for an observational study to evaluate skeletal muscle 11βHSD1 activity in CKD and its relation to skeletal muscle health

Analysis of the RIISC cohort dataset relied on indirect measures of 11βHSD1 activity and indirect measures of sarcopenia risk factors. To investigate associations between 11βHSD1 activity in skeletal muscle and skeletal muscle phenotype in human CKD directly, it was necessary to carry out a new purpose-specific clinical study. The following section describes the findings from the observational study Steroid Hormone Metabolism and Muscle Loss in Chronic Kidney Disease (SMK study; ISRCTN15497320).

The SMK study recruited 17 patients with CKD and 14 healthy volunteers between the ages of 60 to 80 years. Participants' demographic and clinical characteristics are summarised in Table 5.4. In brief, the CKD and healthy control groups are matched for age, gender and body mass index, but clearly separated in terms of kidney function, prevalence of hypertension, haemoglobin concentration, albumin concentration and therapy with anti-hypertensive, alkali and vitamin D medications. Co-existing diabetes mellitus was present in 4 of 17 patients with CKD, but none of the healthy volunteers.

A range of measurements were collected to obtain a detailed skeletal muscle phenotype for all participants. Time taken for five repeated chair rises is higher in the CKD group than the control group, indicating worse lower limb muscle strength (median [interquartile range] 12.1sec [9.1sec - 14.7sec] and 9.4sec [7.1sec – 10.7sec] for CKD and healthy group respectively, p=0.010, Figure 5.3B). This difference remains statistically significant in a sensitivity analysis when data from two patients who were unable to complete this task is censored. Overall

Table 5.4 Participant characteristics for the SMK study

	Healthy volunteer	CKD patients	p-value
n	14	17	
Age (years), mean ± s.d.	71.9 ± 5.1	69.8 ± 5.1	0.264
Male, n (%)	8 (57%)	10 (59%)	0.999
Ethnicity			
White, n (%)	12 (86%)	9 (53%)	0.052
Asian, n (%)	1 (7%)	3 (18%)	
Black, n (%)		4 (23%)	
Unknown, n (%)	1 (7%)	1 (6%)	
Aetiology of renal disease			
Diabetes, n (%)	n/a	3 (18%)	
Ischaemic/hypertensive, n (%)		2 (12%)	
Glomerular disease, n (%)		3 (18%)	
Hereditary, n (%)		1 (6%)	
Obstructive, n (%)		1 (6%)	
Interstitial disease, n (%)		1 (6%)	
Other, n (%)		2 (12%)	
Unknown, n (%)		4 (24%)	
Comorbidities			
Diabetes mellitus, n (%)	0	4 (24%)	0.052
Hypertension, n (%)	5 (36%)	15 (88%)	0.002
Other CVD, n (%)	0	2 (12%)	0.185
Medications			
Antihypertensive count, median [IQR]	0 [0 - 1]	2 [0 - 4]	0.026
Bicarbonate, n (%)	0	6 (35%)	0.013
Vitamin D, n (%)	1 (7%)	9 (53%)	0.007
BMI (kg/m^2), mean ± s.d.	27.4 ± 4.1	27.0 ± 3.5	0.8244
eGFR (mL/min/1.73m^2), mean ± s.d.	81.4 ± 11.5 (n=11)	16.8 ± 5.62	<0.0001
urine ACR (mg/mmol), median [IQR]	no data	35.8 [10.6 - 108.4]	-
Haemoglobin (g/L), mean ± s.d.	140 ± 18.5 (n=9)	116 ± 13.9	<0.001
tCO_2 (mmol/L), mean ± s.d.	no data	21.4 ± 1.5	-
HbA_{1c}(mmol/mol), mean ± s.d.	38.6 ± 2.2 (n=10)	38.4 ± 4.8 (n=14)	0.884
Albumin (g/L), mean ± s.d.	41.4 ± 1.4 (n=8)	36.9 ± 5.7	0.041

Comparisons by unpaired t-test for continuous variables and Fisher's exact test for categorical variables. Non-white ethnicities were pooled as one category for comparative analysis.
s.d., standard deviation; CVD, cardiovascular disease; BMI, body mass index; eGFR, estimated glomerular filtration rate based on creatinine-derived CKD-EPI equation; ACR, albumin-creatinine ratio; tCO_2, total serum bicarbonate; HbA_{1c}, glycated haemoglobin.

physical function is also worse in the CKD group than in the control group, as demonstrated by a lower overall score in the short physical performance battery (SPPB) (median [IQR] 10 [7.5-12] and 12 [11-12] for CKD and healthy group respectively, p=0.006, Figure 5.3I). By themselves as components of the SPPB score, the trends for slower gait speed (0.922±0.303m/s and 1.030±0.204m/s for CKD and healthy group respectively, p>0.05, Figure 5.3G) and impaired standing balance (Figure 5.3H) in the CKD group compared to healthy volunteers do not reach statistical significance. Maximal grip strength in the dominant hand is similar in the CKD and control groups (29.5±10.5kg and 30.0±8.8kg for CKD and healthy group respectively, p>0.05, Figure 5.3A). No difference is evident in absolute or normalised measures of appendicular muscle mass (Figure 5.3D-F). According to EWGSOP2 criteria, 5 of 17 patients with CKD and 1 of 14 healthy volunteers have probable sarcopenia, but no participants have confirmed sarcopenia. In conclusion, risk factors for sarcopenia are more common in the CKD group with reduced lower limb strength and worse overall physical function compared to the healthy control group, but no study participant meets the diagnostic criteria for overt sarcopenia.

Self-reported information on physical activity and malnutrition risk was collected to evaluate potential biases between groups or identify outliers. Generally speaking, patients with CKD and healthy volunteers report equivalent time spent doing vigorous, moderate or walking activities, as well as equivalent time spent seated (Figure 5.4A&B). Moderate risk for malnutrition is present in 1 healthy volunteer and 1 patient with CKD, and mild risk for malnutrition is present in 3 patients with CKD, according to the short nutritional assessment questionnaire (Figure 5.4C). Taken together, the data does not indicate substantial differences in physical activity or nutritional status between the two study groups.

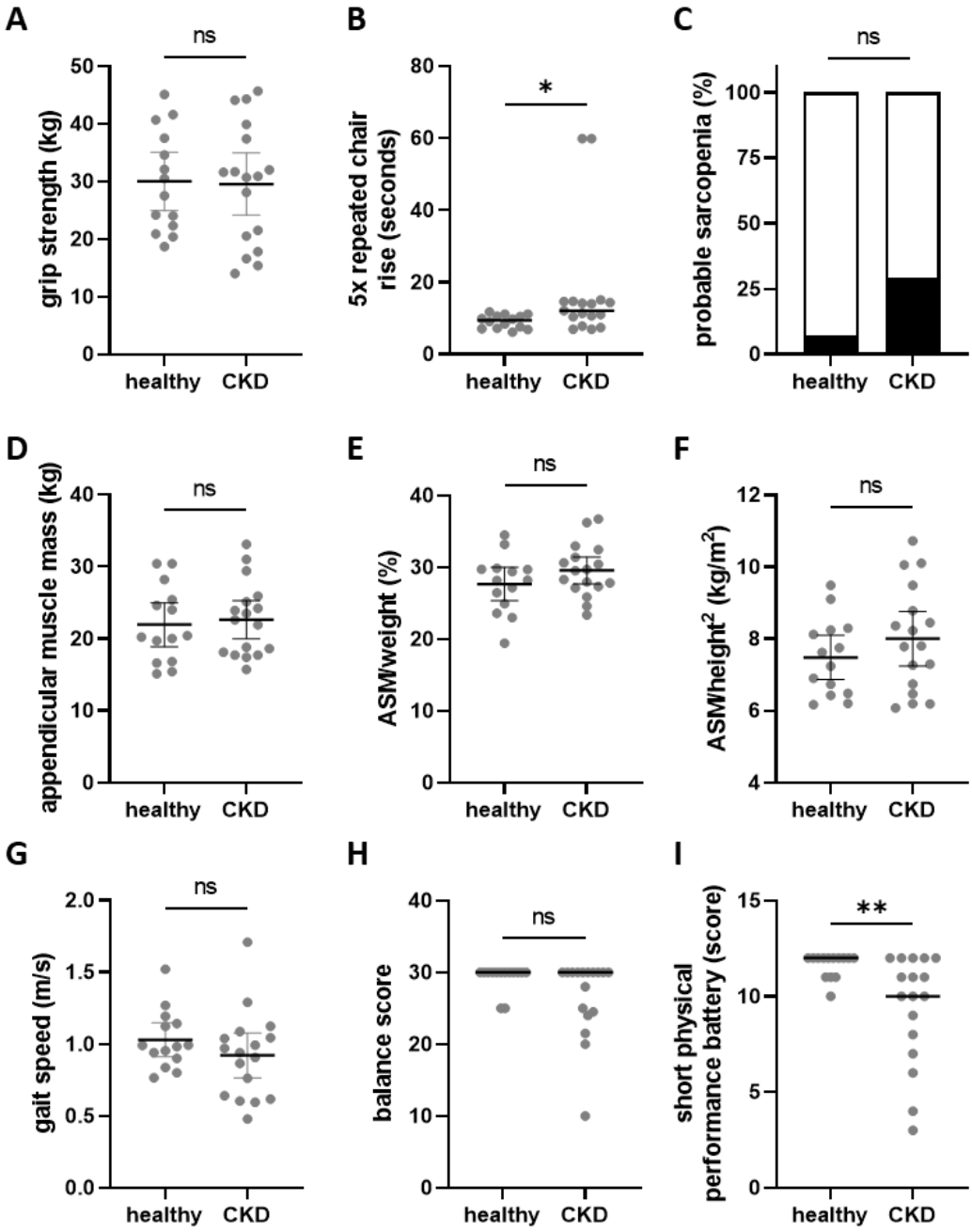


Figure 5.3 Skeletal muscle phenotype for people with CKD and healthy volunteers in the SMK study.

A) *Maximal grip strength in the dominant hand based on three attempts.* ***B)*** *Time taken for completing five consecutive chair rises; data censored at 60 seconds.* ***C)*** *Proportion of participants in each group with probable sarcopenia according to the EWGSOP2 definition.[14] Analysis with Fisher's exact test.* ***D)*** *Appendicular skeletal muscle mass (ASM) as estimated by bioelectrical impedance analysis.* ***E)*** *ASM normalised to body weight.* ***F)*** *ASM normalised to the square of body height.* ***G)*** *Fasted gait speed over 4 meters based on two attempts.* ***H)*** *Cumulative time for holding balance in three different standing position, with cut-ff at 10*

seconds for each position, as per the Short Physical Performance Battery. ***I)*** *Cumulative score from the Short Physical Performance Battery. Normally distributed data is shown with means (solid line) and 95% confidence intervals (error bars), with comparisons using unpaired t-tests. Data with a skewed distribution is shown with median (solid line), with comparisons using Mann-Whitney U tests. ns p>0.05, * p<0.05, ** p<0.01. [legend continued from previous page]*

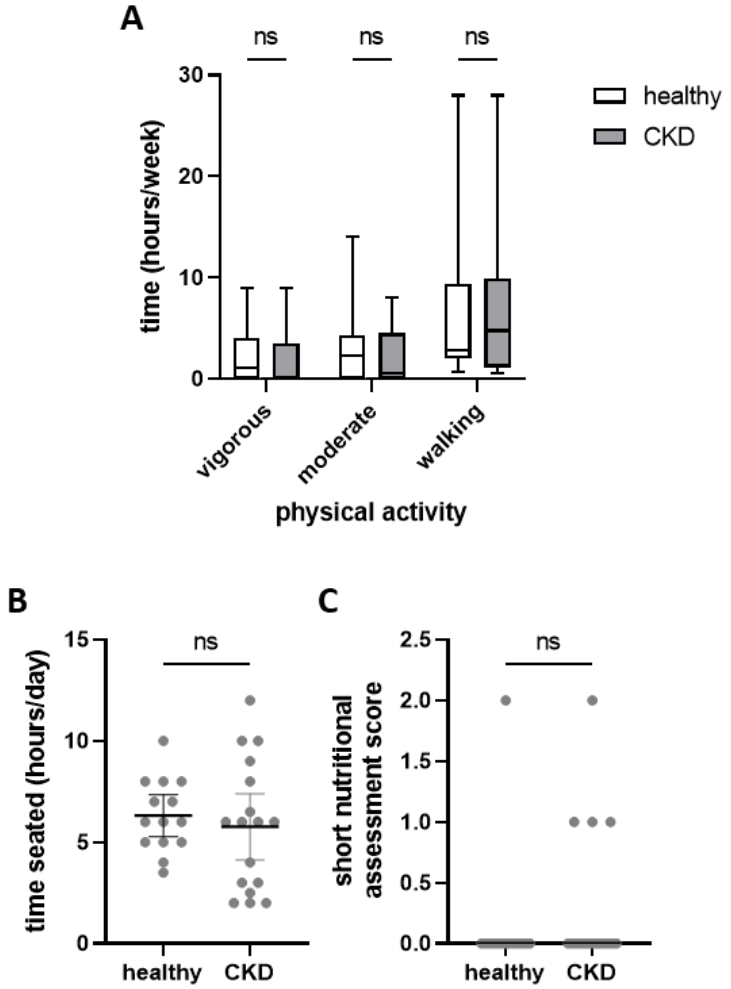


Figure 5.4 Physical activity levels and malnutrition risk for participants in the SMK study. ***A)*** *Participant-reported time spent doing vigorous, moderate and walking activity per week as per the International Physical Activity Questionnaire (IPAQ). Box and whiskers indicate median, inter-quartile range and 5th to 95th percentile; comparisons by Mann-Whitney U test.* ***B)*** *Participant-reported time spent seated per usual weekday as per IPAQ. Data shown with means and 95% confidence intervals, comparison using unpaired t-test.* ***C)*** *Malnutrition risk score as per the Short Nutritional Assessment Questionnaire (0-1 no/mild malnutrition risk; 2 moderate malnutrition risk; ≥3 severe malnutrition risk). Comparison using Fisher's exact test with pooled frequencies for scores ≥1. ns p>0.05.*

5.2.4 11βHSD1 enzymatic activity in skeletal muscle is similar in people with and without CKD

A primary objective of the SMK study was to investigate 11βHSD1 activity in skeletal muscle of patients with CKD in comparison to healthy volunteers. To this end, fresh quadriceps muscle tissue biopsy samples were incubated with radiolabelled cortisone ex vivo for 24 hours to measure 11βHSD1 enzymatic rate of cortisol activation directly. Mean 11βHSD1 enzymatic rate in muscle from patients with CKD is 107.5 ±53.6 fmol/mg protein/24h and mean 11βHSD1 enzymatic rate in muscle from healthy volunteers is 101.1 ±22.5 fmol/mg protein/24h ($p>0.05$ in independent t-test, Figure 5.5). The mean difference between groups is 6.4 fmol/mg protein/24h (95% C.I. -23.3 to 36.1 fmol/mg protein/24h), representing a percentage difference of 6.3%. In short, 11βHSD1 activity in skeletal muscle in this study is closely matched between people with and without CKD.

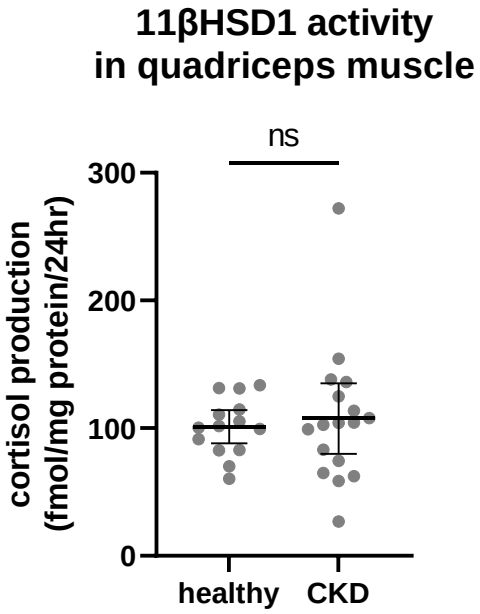


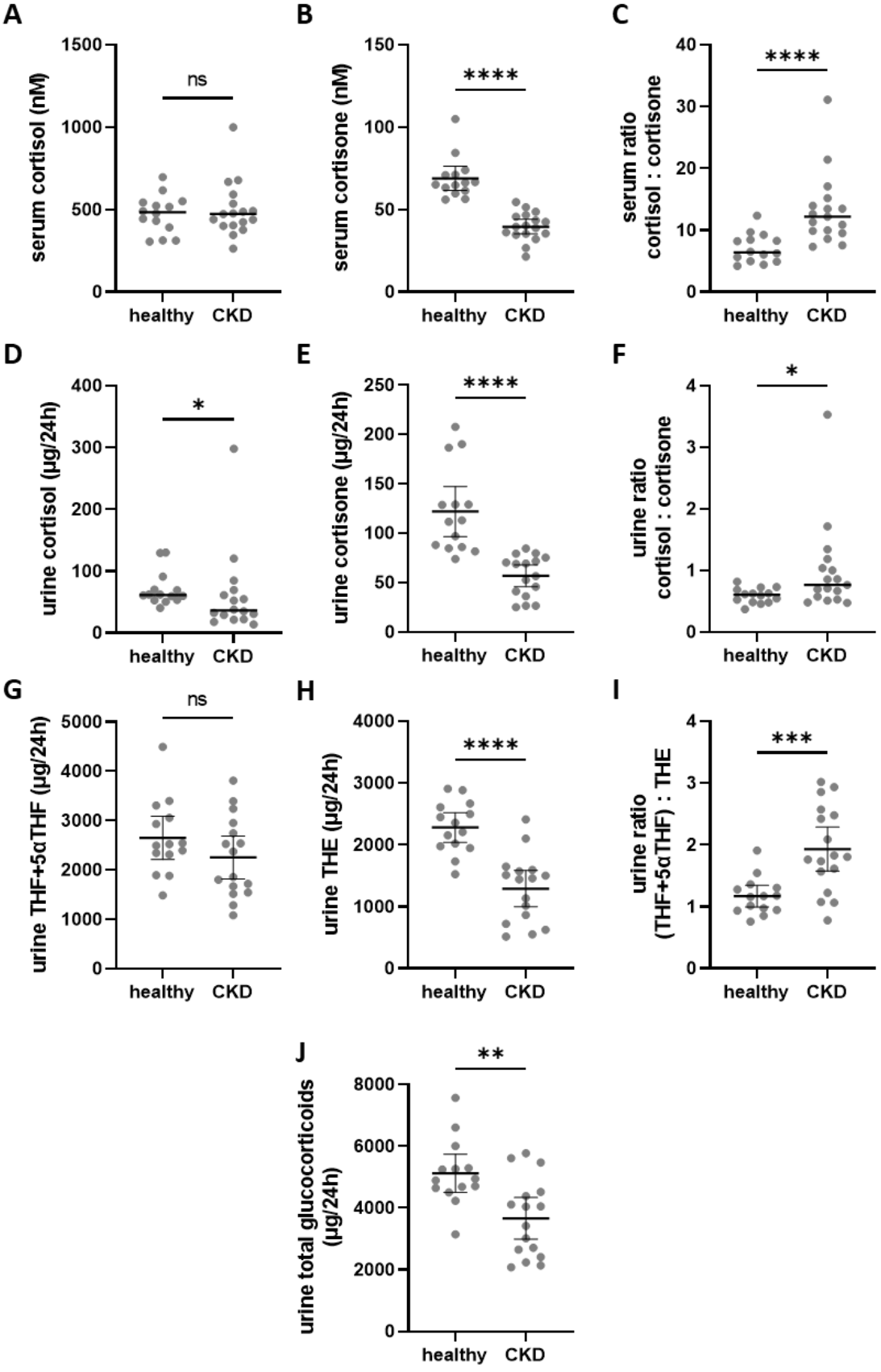
Figure 5.5 Enzymatic activity of 11βHSD1 in quadriceps muscle biopsies from patients with CKD and healthy volunteers.
Enzymatic activity was measured ex vivo as the conversion of inactive cortisone to active cortisol in serum-free media at 37^{o}C over 24h, normalised to wet tissue weight. Comparison by unpaired t-test; ns $p>0.05$.

5.2.5 Inactive glucocorticoid levels are reduced in CKD with little change in active glucocorticoid levels

Cortisol, cortisone and their metabolites were measured in fasted 8am blood samples and in 24-hour urine samples to put the results of skeletal muscle 11βHSD1 activity in the context of systemic glucocorticoid shifts in CKD. Early morning serum cortisol levels are similar in CKD and healthy control groups (median [IQR] 473nM [402-564nM] and 484nM [373-545nM] for CKD and healthy groups respectively, $p>0.05$, Figure 5.6A). In contrast, early morning serum cortisone levels are markedly lower in the CKD group than in the control group (39.6±8.6nM and 69.0±12.7nM for CKD and healthy groups respectively, $p<0.0001$, Figure 5.6B). Consequently, the ratio of active cortisol to inactive cortisone in early morning blood samples is significantly higher in the CKD group than in the control group ($p<0.0001$, Figure 5.6C).

Figure 5.6 Serum and urine glucocorticoid profile for patients with CKD and healthy volunteers.

[figure on following page] Data from the SMK study. ***A)*** *Early morning serum cortisol concentration.* ***B)*** *Early morning serum cortisone concentration.* ***C)*** *Ratio of cortisol to cortisone in early morning serum samples.* ***D)*** *Amount of cortisol in 24-hour urine samples.* ***E)*** *Amount of cortisone in 24-hour urine samples.* ***F)*** *Ratio of cortisol to cortisone in 24-hour urine samples.* ***G)*** *Amount of cortisol metabolites tetrahydrocortisol (THF) plus 5α-tetrahydrocortisol (5αTHF) in 24-hour urine samples.* ***H)*** *Amount of cortisone metabolite tetrahydrocortisone (THE) in 24-hour urine samples.* ***I)*** *Ratio of (THF+5αTHF) to THE in 24-hour urine samples.* ***J)*** *Total amount of glucocorticoids and their metabolites (cortisol + cortisone + THF + 5αTHF + THE) in 24-hour urine samples. Data from the SMK study. Normally distributed data is shown with means (solid line) and 95% confidence intervals (error bars), with comparisons using unpaired t-tests. Data with a skewed distribution is shown with median (solid line), with comparisons using Mann-Whitney U tests. ns $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.*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CKD
I
urine ratio (THF+5αTHF) : THE
4
3
2
1
0
***
healthy
CKD
J
urine total glucocorticoids (µg/24h)
8000
6000
4000
2000
0
**
healthy
CKD

A small proportion of circulating cortisol is excreted unchanged in urine. In this study, free cortisol levels in 24-hour urine samples are lower in the CKD group than in the control group (median [IQR] 36.5μg/24h [23.8-67.3μg/24h] and 61.6μg/24h [53.3-75.6μg/24h] for CKD and healthy groups respectively, p=0.031, Figure 5.6D). A more pronounced shift in the same direction is also evident for free cortisone levels in 24-hour urine samples (56.8±20.8μg/24h and 121.9±43.8μg/24h for CKD and healthy groups respectively, p<0.0001, Figure 5.6E). In line with these changes, the ratio of cortisol to cortisone is slightly higher in people with CKD than healthy volunteers (p=0.021, Figure 5.6F).

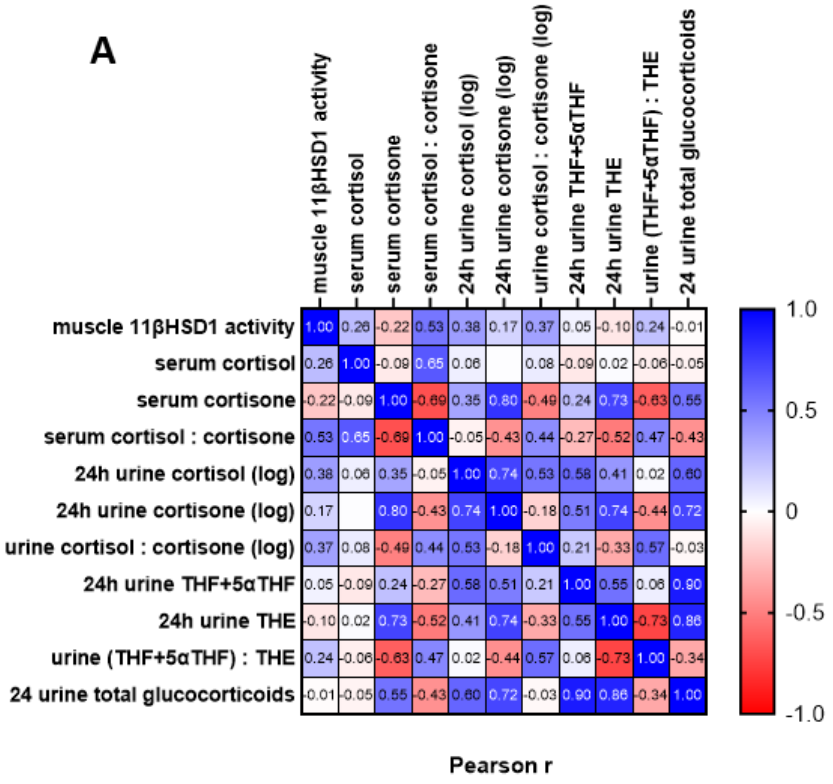
The majority of cortisol in circulation is metabolised in the liver prior to urinary excretion as THF or 5αTHF metabolites, while cortisone is excreted predominantly in the form of THE. Total urinary excretion of THF + 5αTHF over 24 hours is not significantly different in the CKD and control groups (2253±815μg/24h and 2647±759μg/24h for CKD and healthy groups respectively, p>0.05, Figure 5.6G). In contrast, total urinary excretion of THE over 24 hours is significantly lower in the CKD group than in the control group (1292±551μg/24h and 2281±417μg/24h for CKD and healthy groups respectively, p<0.0001, Figure 5.6H). Accordingly, the ratio of (THF+5αTHF)/THE is elevated in patients with CKD compared to healthy volunteers (p<0.001, Figure 5.6I). It is worth highlighting that the pattern of changes in urinary metabolites of cortisol and cortisone mirrors the pattern of changes in serum cortisol and serum cortisone. While serum cortisol and urinary cortisol metabolites change little in CKD, significant reductions are evident for serum cortisone and urinary cortisone metabolites.

Finally, the sum of all glucocorticoids and their metabolites in a 24-hour urine sample is often regarded as an indicator of cumulative systemic glucocorticoid exposure. In this study, total glucocorticoid content (cortisol + cortisone + THF + 5αTHF + THE) in 24-hour urine samples is significantly lower in the CKD group than in the control group (3663±1267μg/24h and 5121±1066μg/24h for CKD and healthy groups respectively, p=0.002, Figure 5.6J).

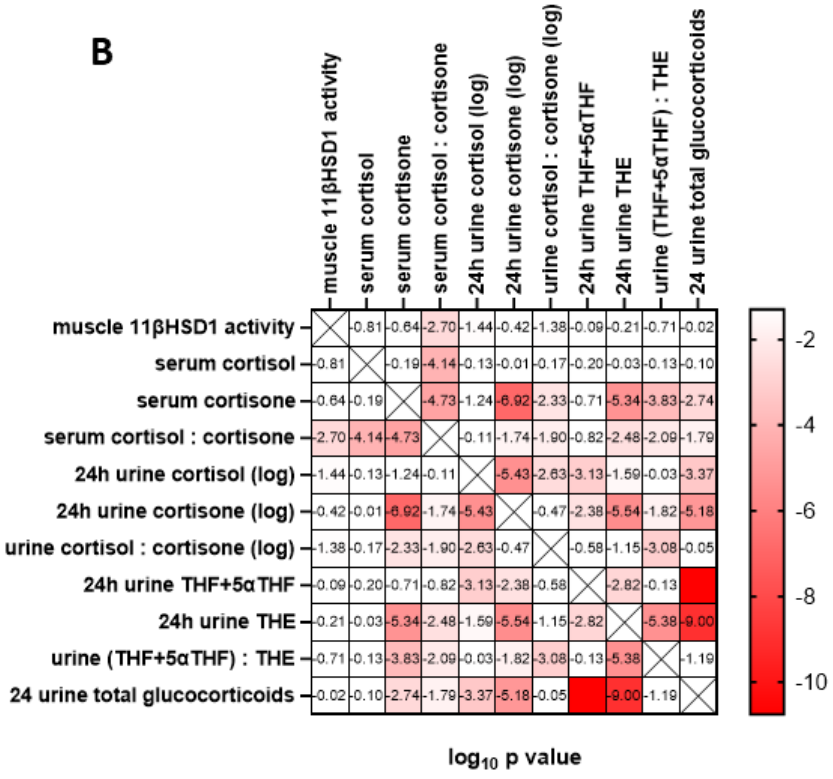
Having gathered data on a range of glucocorticoid indices, it is possible to examine the extent of correlation between different glucocorticoid indices in this study. An exploratory analysis of this kind can evaluate the validity of surrogate markers for skeletal muscle 11βHSD1 activity and other aspects of glucocorticoid physiology. Data from participants with and without CKD were pooled for this analysis. Interestingly, skeletal muscle 11βHSD1 activity correlates most closely with serum cortisol/cortisone ratio (Pearson r= 0.53, p=0.002 without adjustment for multiple comparisons), but the correlation with urine (THF+5αTHF)/THE ratio is only weak to moderate (Pearson r= 0.24, unadjusted p>0.05). Another noteworthy observation is a lack of correlation for early morning serum cortisol with any glucocorticoid measure in urine (Figure 5.7). In contrast, serum cortisone correlates strongly with urine cortisone, urine THE, urine (THF+5αTHF)/THE ratio, and moderately with total urinary glucocorticoid excretion. These data suggest that measurements of glucocorticoids in urine may not provide an accurate reflection of tissue-specific 11βHSD1 activity or circulating cortisol levels in populations that include people with CKD.

Figure 5.7 Correlations between indices of glucocorticoid status.

A) *Correlation matrix for measures of 11βHSD1 activity in quadriceps muscle biopsies, measures of glucocorticoids in serum and in 24-hour urine samples. Numbers indicate Pearson correlation coefficient r for the respective row – column pair of glucocorticoid indices. Colours are graded according to the number scale on the right-hand side. 24h urine cortisol values, 24h urine cortisone values and 24h urine cortisol : cortisone ratios were transformed with the log function for this analysis. Data from the CKD group and healthy control group of the SMK study has been pooled for this analysis; total n=31.*



B) *Corresponding p values for the correlation matrix shown in A), with p values transformed using the log_{10} function. An unadjusted p value = 0.05 corresponds to $log_{10}(0.05)$ = -1.30; a p value adjusted for 55 multiple comparisons using the conservative Bonferroni method of 0.00091 corresponds to $log_{10}(0.05/55)$ = -3.04. Colours are graded according to the number scale on the right-hand side.*



5.2.6 Skeletal muscle 11βHSD1 activity correlates with muscle strength and function independent of CKD status

A secondary objective in the SMK was the investigation of 11βHSD1 activity in skeletal muscle in relation to muscle mass, strength and function. Given the lack of a difference 11βHSD1 activity in skeletal muscle from participants with and without CKD, data from both groups is pooled for this analysis to maximise statistical power. Skeletal muscle 11βHSD1 correlates negatively with grip strength in the study cohort (Pearson r= -0.466, p=0.008, Figure 5.8A). Moreover, skeletal muscle 11βHSD1 correlates negatively with gait speed as a measure of muscle function (Pearson r= -0.374, p=0.038, Figure 5.8D). Further trends suggest possible associations of skeletal muscle 11βHSD1 activity with reduced lower limb strength as measured by the repeated chair-rise test (Spearman rho= 0.351, p=0.052, Figure 5.8B) and poorer general physical function as measured by the SPPB (Spearman rho= -0.308, p>0.05, Figure 5.8E), although these trends fall short of reaching the pre-specified cut-off for statistical significance. In a post-hoc analysis of the separate study groups, the association between muscle 11βHSD1 activity and grip strength remained statistically significant in the group of healthy volunteers (Pearson r= -0.570, p=0.033, Figure 5.8F). The level of statistical significance for the same association in the group of patients with CKD is marginally less (Pearson r= -0.460, p=0.063, Figure 5.8F). There are no associations of muscle 11βHSD1 activity with any measure of body composition in this study, not with measures of lean tissue mass nor with measures of adiposity (Figure 5.8C & Figure 5.10). Taken together, 11βHSD1 activity in skeletal muscle exhibits significant correlations or consistent trends for associations with impaired muscle strength and function, but not with reduced muscle mass.

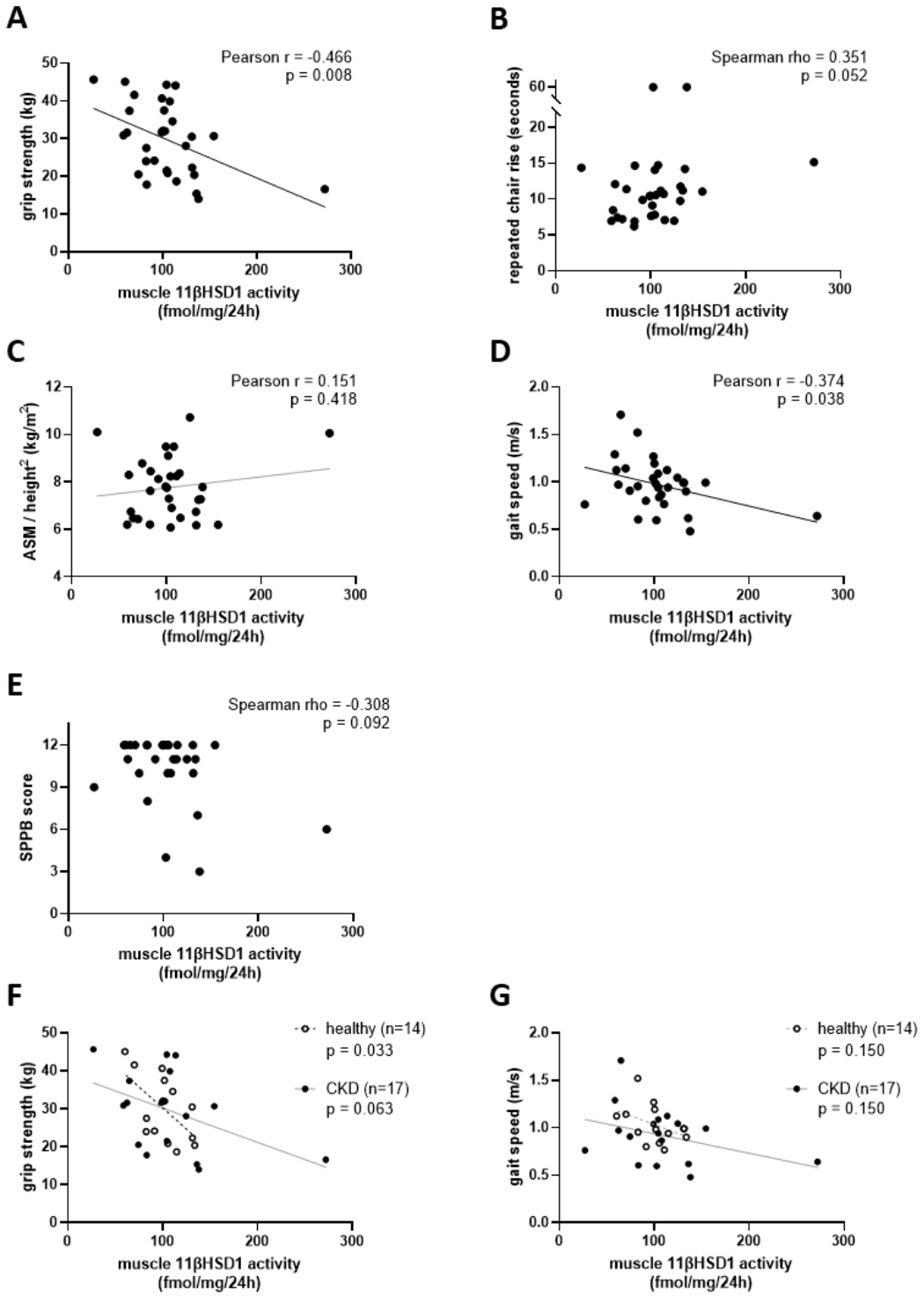


Figure 5.8 Correlations between 11ϐHSD1 activity in quadriceps muscle biopsies and measures of muscle strength, mass and function.

A-E) *Correlation analysis for 11ϐHSD1 activity in quadriceps muscle biopsies and maximal grip strength in the dominant hand* ***(A)****, time taken for 5 consecutive chair rises (censored at 60sec)* ***(B)****, appendicular skeletal muscle mass normalised to the square of body height* ***(C)****, gait speed over 4 meters* ***(D)****, and total score with the Short Physical Performance Battery* ***(E)****. Data from*

the CKD group and healthy volunteer group of the SMK study were pooled for analysis A) – E) (n=31). Correlation analysis using Pearson's r or Spearman's rho (for variables with a skewed data distribution). Solid lines indicate simple linear regression fit, where appropriate. ***F-G)*** *Pearson correlation analysis for 11ϐHSD1 activity in quadriceps muscle biopsies and grip strength* ***(F)*** *or gait speed* ***(G)****, with separate analysis for the CKD group (n=17, solid line indicating simple linear regression fit) and the healthy volunteer group (n=14, dashed line indicating simple linear regression fit). In a sensitivity analysis with exclusion of the high outlier in the 11ϐHSD1 activity data, the correlation with grip strength remained statistically significant. [legend continued from previous page]*

To explore the relationship between muscle 11βHSD1 activity and grip strength further, several potential confounding variables were evaluated. Despite the age restriction for the inclusion criteria of the study, participant age still correlates strongly with 11βHSD1 enzymatic activity in skeletal muscle tissue (Pearson r=0.559, p=0.001, Figure 5.9A). The association with age appears equally strong regardless of CKD status (CKD group: Pearson r= 0.665, p=0.004, n=17; healthy group: Pearson r= 0.544, p=0.044, n=14; Figure 5.9B) or gender (men: Pearson r=0.561, p=0.016, n=18; women: Pearson r=0.690, p=0.011, n=13). No significant associations with muscle 11βHSD1 activity are observed for gender, body mass index or diabetes diagnosis (Table 5.5). Grip strength is associated with gender, and possibly with age, but not with body mass index or diabetes diagnosis in the study cohort (Table 5.6). Based on these results, a multivariable linear regression was generated with grip strength as outcome variable and age, gender, CKD status and muscle 11βHSD1 activity as independent variables. In this model, muscle 11βHSD1 activity alongside gender remain independently associated with grip strength (Table 5.7). The data therefore support a correlation between elevated 11βHSD1 enzymatic activity in skeletal muscle tissue and lower muscle strength or increased sarcopenia risk. Also, age is an important predictor of elevated 11βHSD1 activity in skeletal muscle.

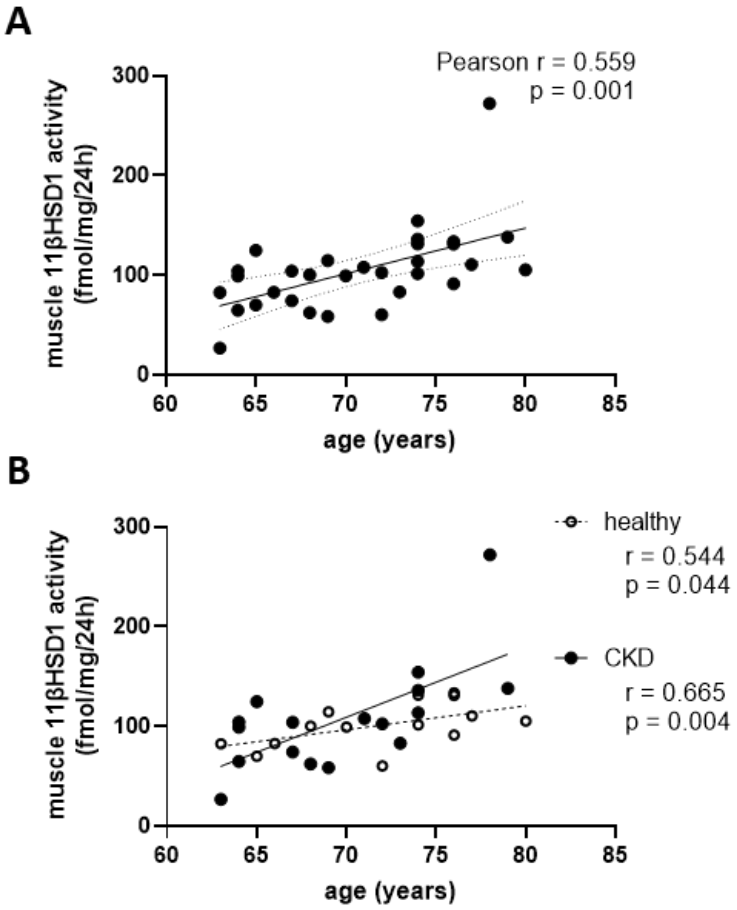


Figure 5.9 Correlation between age and 11ϐHSD1 activity in quadriceps muscle biopsies. ***A)*** *Pearson correlation analysis for participant age and 11ϐHSD1 activity in quadriceps muscle biopsies, with pooled data from the CKD group and healthy volunteer group of the SMK study (n=31). Solid line indicates simple linear regression fit, dotted curves indicate 95% confidence interval for regression line.* ***B)*** *Pearson correlation analysis for participant age and 11ϐHSD1 activity in quadriceps muscle biopsies, with separate analysis for the CKD group (n=17, solid line indicating simple linear regression fit) and the healthy volunteer group (n=14, dashed line indicating simple linear regression fit) of the SMK study. In a sensitivity analysis with exclusion of the high outlier in the 11ϐHSD1 activity data, all correlations remained statistically significant.*

Table 5.5 Exploration of potential confounding associations with 11βHSD1 activity in quadriceps muscle biopsies

Variable	p values all (n=31)	HC (n=14)	CKD (n=17)
Age	0.001	0.044	0.004
Male gender	0.601	0.309	0.351
Body mass index	0.967	0.320	0.615
Diabetes mellitus	0.302	-	0.278

Analysis using Pearson correlation for continuous variables and unpaired t-test for categorical variables. Analysis was performed for pooled CKD and healthy control groups, as well as within each group separately. HC, healthy control; CKD, chronic kidney disease.

Table 5.6 Exploration of potential confounding associations with grip strength

Variable	p values all (n=31)	HC (n=14)	CKD (n=17)
Age	0.058	0.487	0.06
Male gender	<0.001	<0.001	0.072
Body mass index	0.958	0.200	0.292
Diabetes mellitus	0.109	-	0.206

Analysis using Pearson correlation for continuous variables and unpaired t-test for categorical variables. Analysis was performed for pooled CKD and healthy control groups, as well as within each group separately. HC, healthy control; CKD, chronic kidney disease.

Table 5.7 Multivariable linear regression model for grip strength

		95% C.I. for B				
Variable	**B**	**lower**	**upper**	**β**	**t**	**p**
Age (year)	-0.141	-0.694	0.413	-0.075	-0.521	0.606
Male gender	12.091	7.393	16.788	0.631	5.281	<0.001
Muscle 11βHSD1 activity (fmol/mg/24h)	-0.111	-0.179	-0.044	-0.486	-3.37	0.002

Adjusted R^2=0.578. Model based on pooled data of CKD group and healthy volunteer group from SMK study, n=31.

In a sensitivity analysis, swapping age for CKD status as an independent variable does not substantially alter the model parameters.

5.2.7 Skeletal muscle 11βHSD1 activity is more closely correlated with grip strength and gait speed than alternative indices of glucocorticoid balance

In a final exploratory analysis, associations of systemic glucocorticoid indices with skeletal muscle phenotype were investigated across the pooled SMK study cohort. The results show that muscle 11βHSD1 activity correlates as well or better with grip strength, gait speed and chair-rise speed than early morning serum cortisol, THF+5αTHF excretion in 24-hour urine samples or urinary (THF+5αTHF)/THE ratio (Figure 5.10). Early morning cortisol exhibits a trend for an association with lower normalised muscle mass, but this trend does not reach statistical significance in the pooled study cohort even before adjustment for multiple comparison testing. Finally, data from this study indicates that urinary (THF+5αTHF)/THE ratio is weakly to moderately correlated with measures of compound muscle function such as gait speed, repeated chair rises and cumulative SPPB score, but not with grip strength as an isolated measure of forearm muscle strength. While these findings can be informative, they remain exploratory in nature and should be interpreted with caution.

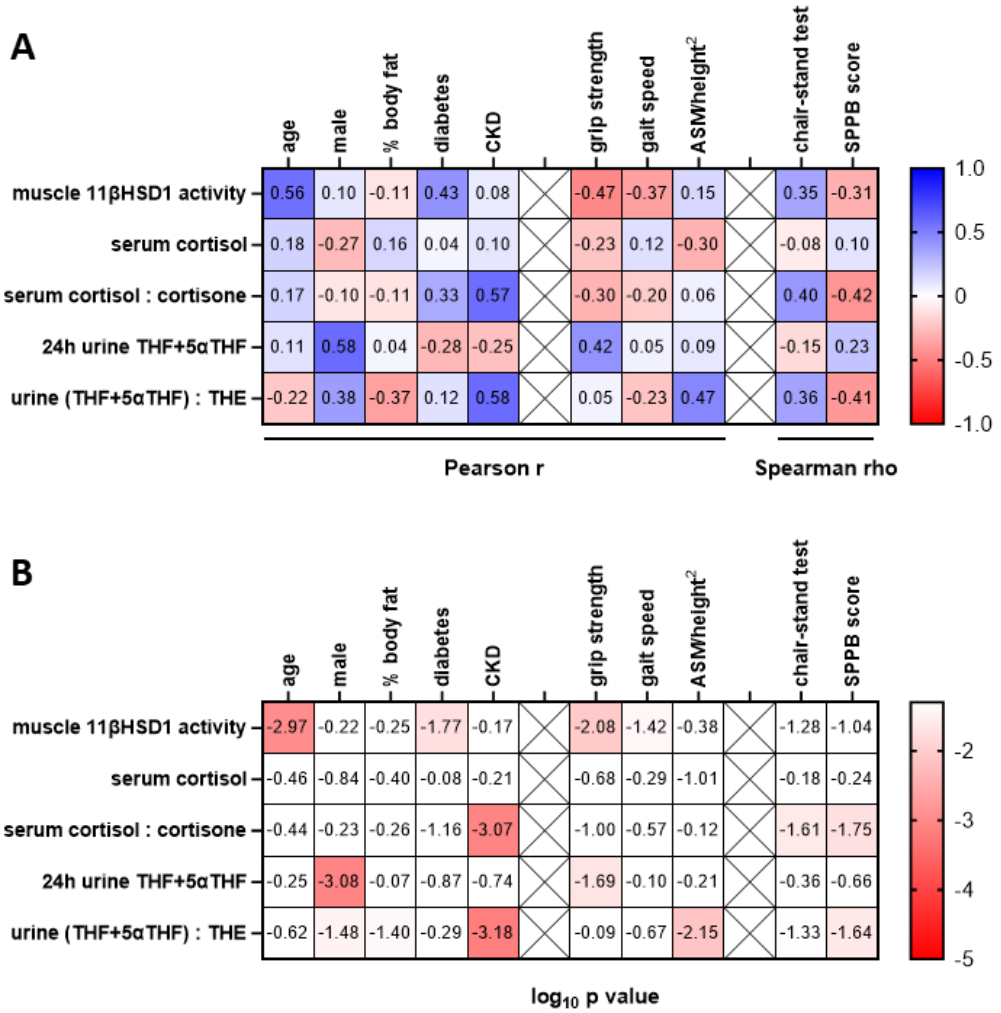


Figure 5.10 Correlations between glucocorticoid indices, potential confounders and measures of skeletal muscle phenotype.

A) *Correlation matrix for selected indices of glucocorticoid status, potential confounding variables and measures of muscle strength, function and mass. Numbers indicate Pearson correlation coefficient r or Spearman correlation coefficient rho (for variables with a skewed data distribution) for the respective row – column pair. Colours are graded according to the number scale on the right-hand side. Data from the CKD group and healthy control group of the SMK study has been pooled for this analysis; total n=31.* ***B)*** *Corresponding p values for the correlation matrix shown in A), with p values transformed using the log_{10} function. An unadjusted p value = 0.05 corresponds to $log_{10}(0.05)$ = -1.30; a p value adjusted for 25 multiple comparisons using the conservative Bonferroni method of 0.002 corresponds to $log_{10}(0.05/25)$ = -2.7. Colours are graded according to the number scale on the right-hand side.*

5.3 Discussion

This component of the research investigated variation of 11βHSD1 function in skeletal muscle tissue from humans with and without chronic kidney disease. Furthermore, the associations between muscle and systemic measures of 11βHSD1 activity with indicators of muscle mass, strength and function were explored. Ex vivo 11βHSD1 enzymatic activity in quadriceps muscle biopsies was similar for people with CKD and age-matched healthy volunteers. Even within the narrow age band of 60 to 80 years, a correlation of muscle 11βHSD1 activity with age was clearly evident. Additionally, muscle 11βHSD1 activity correlated with grip strength, independent of gender and age. The glucocorticoid metabolite ratio (THF+5aTHF)/THE in urine has limited accuracy as a surrogate marker of 11βHSD1 activity specifically in skeletal muscle. To summarise the main findings, age appears to be more important as a determinant of 11βHSD1 activity in skeletal muscle than kidney function, and the data suggest an association of 11βHSD1 activity in skeletal muscle with muscle strength.

5.3.1 11βHSD1 activity in skeletal muscle in CKD and health

An original case-control study was performed to examine the hypothesis that 11βHSD1 activity in skeletal muscle tissue is elevated in people with chronic kidney disease compared to healthy people. The primary outcome measure of cortisol activation rate in quadriceps biopsies ex vivo was not significantly different between the two groups. Post-hoc analysis showed the relative difference for the CKD group to be small at +6.3%. For a critical interpretation of this finding, the following discussion will scrutinise the validity of the outcome measure, characteristics of the study groups, sample size and scientific context.

The study employed a methodology for quantification of the outcome measure that had appropriate specificity and sensitivity for addressing the research question. Assay specificity for the enzymatic reaction of cortisol activation was achieved through inclusion of radiolabelled cortisone substrate. Conducting the assay on skeletal muscle tissue ex vivo conferred specificity for this tissue type. The assay precision was acceptable with a mean coefficient of variation of 15.9% for duplicate samples. The same assay approach has been validated in the literature by multiple research groups.[330, 351] Additionally, the assay procedure had sufficient accuracy in this study cohort to detect changes related to age, which fits with previous observations on mRNA expression of 11βHSD1 in skeletal muscle.[193] Arguably, the ex vivo testing environment may have influenced results. Alternative approaches for human in vivo testing of 11βHSD1 activity have been described, but these are invasive with greater risks to participants and have inferior specificity for tissue type and enzymatic reaction.[190] It is therefore reasonable to contend that the outcome measure was determined with the best available methodology.

The study design and participant recruitment were optimised for addressing the research question. A case-control study represented the most efficient design for addressing the research question. Study groups were clearly distinct for kidney function and CKD status. At the same time, study groups were well matched for potential confounders of age, gender and body mass index. Diabetes mellitus, which was only represented in the CKD group, was associated with a non-significant trend for elevated muscle 11βHSD1 activity. Thus, diabetes mellitus can be excluded as a factor that may have obscured a true elevation of muscle 11βHSD1 activity in CKD in this study. Still, bias from other sources remains a theoretical

possibility in a case-control study, e.g. due to slight differences in ethnic representation or other unknown confounders. It should also be acknowledged that the CKD group – despite being constituted by patients with advanced renal impairment – was sampled from the ambulatory pre-dialysis population. People with CKD on renal replacement therapy or with impaired physical function may have different levels of 11βHSD1 activity in skeletal muscle, but it would be more challenging to attribute such a difference to reduction in kidney function alone. Therefore, the study design and participant recruitment meet important criteria for addressing the research question in a reliable fashion.

Representation of the sarcopenia phenotype in the SMK study deserves separate consideration as a potential limitation. Previous meta-analyses have suggested sarcopenia prevalence of 10-20% in the aging general population.[15, 16] No participant in the SMK study met the criteria for confirmed sarcopenia according to the EWGSOP2 definition. Criteria for probable sarcopenia were reached for 7% in the control group and 29% in the CKD group. The lower degree of sarcopenia observed in the SMK cohort overall is in keeping with other research cohorts composed of ambulant voluntary responders. It reflects a recruitment bias towards healthier members of the population of interest.[26] This bias could reduce the capacity of the study to detect differences in muscle 11βHSD1 between the CKD and control group, especially if it affects the study groups unequally. Importantly however, the CKD and control study groups still encapsulate expected relative differences in skeletal muscle phenotype. Physical performance measures such as repeated chair rises or SPPB score are worse in the CKD group. Therefore, differences in underlying risk factors for sarcopenia can reasonably be expected between the study groups. This characteristic of the SMK study

mitigates against the risk of a false negative result due to recruitment bias. Further strategies to address this potential bias would require changes to the recruitment procedure or study design. Such changes are not without drawbacks either. Modifying the recruitment procedure to enhance the representation of sarcopenia in the study carries the risk of introducing new biases and confounders. While it is theoretically possible to investigate the research question with a cohort study design, the required sample size, resources and time would be unduly greater. The conducted study therefore represents a carefully considered approach to the question whether CKD is associated with raised 11βHSD1 activity in skeletal muscle.

Sample size is an important consideration for the interpretation of studies with negative results. The study was planned with a total recruitment target of 50 participants to allow detection of a 34% difference in the primary outcome measure at 80% statistical power and 0.05 statistical significance. These values reflect observations from previous studies and deliberations on a biologically relevant difference.[322, 330, 351] Recruitment to the study had not been completed at the time of preparing this research report, so presented data reflects progress to date and falls short of the intended target. With the current sample size, the post-hoc statistical power for detecting the originally targeted 34% outcome difference is 62.4%. Expressed in an alternative way, it is possible to detect a 42% outcome difference at 80% statistical power with the current sample size. However, when the observed relative difference of 6.3% in the outcome measure is taken as the basis for sample size estimation, a total sample size of 1362 is required to achieve 80% statistical power at 0.05 statistical significance. Expanding the study to this scale presents substantial practical challenges and the biological relevance of a difference that small is dubious.

The final step for interpreting the observed lack of difference in muscle 11βHSD1 activity in CKD and health relies on reviewing this result in the wider scientific context. The major known determinants of 11βHSD1 expression in muscle are inflammation, old age and glucocorticoids themselves.[189, 432] Age and glucocorticoid measures in serum or urine were not increased in the CKD group compared to the healthy group, excluding these factors as potential causes for elevated muscle 11βHSD1 activity. Regulation by inflammatory cytokines is therefore a crucial notion around a hypothesis of increased muscle 11βHSD1 activity in CKD. Ex vivo muscle 11βHSD1 activity correlated with inflammation markers in previous studies, specifically in populations with primary immune-mediated pathology or considerable levels of acute inflammation.[263, 322, 351] In a CKD cohort, serum C-reactive protein correlated with the glucocorticoid metabolite ratio (THF+5aTHF)/THE in urine [320], but this surrogate marker has little specificity for 11βHSD1 activity in skeletal muscle. Unfortunately, characterisation of the inflammation profile is not yet available for the performed study, which will be relevant for the interpretation of results. Based on data from comparable populations, inflammatory cytokines are modestly elevated in ambulatory patients with pre-dialysis CKD, but usually at much lower levels than patients with primary immune-mediated diseases or acute illness.[26, 55, 374, 433] Looking back at results from preclinical models of uraemia in mice (adenine model of renal impairment; chapter 3) and human myotubes (CKD serum condition; chapter 4), inflammatory signalling did not emerge as a relevant or sufficient stimulus in those uraemia models to induce 11βHSD1 upregulation in muscle tissue. These preclinical studies identified no alternative pathway that may upregulate muscle 11βHSD1 in uraemia, either. In the absence of major inflammation, reduction in renal function by itself may therefore not be associated with increased 11βHSD1 activity in skeletal muscle.

To summarise a comprehensive discussion on the observation of similar muscle 11βHSD1 activity in humans with and without CKD, the following conclusions are proposed. First, an appropriate methodology was employed for assessing the outcome variable of interest. Second, the study design and participant recruitment were appropriate for addressing the research question. Third, the sample size was slightly smaller than originally intended, but the estimated effect size based on the available data could be considered too small to justify expansion of the sample size. Finally, the results from this clinical study agree with results from preclinical models that reduction in renal function by itself does not lead to notable upregulation of 11βHSD1 activity in skeletal muscle. Accordingly, the sustained modest rise of circulating inflammatory cytokines which commonly accompanies CKD may not be sufficient on its own to alter 11βHSD1 activity in skeletal muscle. Further data on inflammation phenotype in serum and muscle for the described study is pending and will further inform interpretation of the results. Finally, caution must be advised that these results for muscle tissue cannot be readily generalised for 11βHSD1 activity in other tissues like liver or fat.

5.3.2 Muscle 11βHSD1 activity correlates with age

11βHSD1 activity in skeletal muscle displayed a strong positive correlation with age in the SMK study. This relationship was evident despite the restricted age range of participants from 60 to 80 years, and it was evident independently of CKD status or gender. A positive correlation between 11βHSD1 mRNA expression and age has previously been reported for women, but that study found no clear association for men.[193] The results presented here are original for their validation of an age-dependent increase in biological activity of 11βHSD1 in skeletal muscle, and for demonstrating this correlation in both men and women. Age-dependent rise

in 11βHSD1 activity has already been shown for other human tissues, notably skin, brain, osteoblasts, fat and liver.[434-437] Even though it remains unclear what exactly drives these changes with age, the correlation of age and 11βHSD1 activity in skeletal muscle is consistent with the general pattern described in the literature.

5.3.3 The role of 11βHSD1 activity for muscle phenotype

A secondary objective for this component of the research was exploring the relevance of 11βHSD1 for sarcopenia risk in people with CKD. This question was addressed in two separate study cohorts. The RIISC cohort represents a larger sample of ambulatory people with moderate to advanced CKD. As muscle-specific data has not been collected for that study, the analysis utilised surrogate markers of systemic glucocorticoid metabolism in urine, self-reported indicators of physical function and indirect estimates of lean tissue mass. Many features of the SMK study cohort are complementary to the RIISC cohort. The SMK study included a much smaller sample of aging people with advanced CKD, as well as a matched healthy volunteer group. Collection of muscle-specific data formed an integral part of the rationale for the SMK study, covering both muscle-specific glucocorticoid metabolism measurements and muscle phenotype measurements. Given that muscle 11βHSD1 activity was comparable in participants with and without CKD, the groups were pooled for exploring associations between muscle 11βHSD1 activity and indicators of sarcopenia risk. The following discussion will in turn focus on the chief components of sarcopenia as per the EWGSOP2 definition – muscle strength, muscle function and muscle mass.

5.3.3.1 Muscle strength

11βHSD1 activity in quadriceps biopsies was negatively correlated with grip strength in the SMK study. This association remained statistically significant after adjustment for gender and age of study participants. A similar trend was apparent in both CKD and healthy subgroups, but it only reached statistical significance in the healthy control subgroup, with small sample size presenting a limitation. The data also indicated a trend for higher muscle 11βHSD1 activity correlating with impaired lower limb strength in the repeated chair rise test, but the significance level was borderline with the present sample size. Interestingly, grip strength correlated more strongly with 11βHSD1 activity in quadriceps biopsies than other indices of systemic glucocorticoid balance, such as morning serum cortisol, urinary cortisol metabolite excretion or ratios of active and inactive glucocorticoids. This suggests that muscle 11βHSD1 activity is a more specific predictor of muscle strength than systemic glucocorticoid measures.

Evidence for a negative correlation between 11βHSD1 regulation in skeletal muscle and muscle strength is supported by other human studies. Although no relevant data was available to investigate this relationship in the RIISC cohort, two previous studies in aging populations without CKD provide relevant information. Hassan-Smith et al. report a negative correlation for 11βHSD1 mRNA expression in skeletal muscle and grip strength.[193] Similarly, Kilgour et al. report a negative correlation for 11βHSD1 expression in skeletal muscle and knee strength, independent of height and gender.[331] Despite consistent findings from multiple studies, the evidence remains founded on observational data in humans with inherent risks for confounding. Biological plausibility for amplified glucocorticoid signalling reducing muscle strength is conspicuous [222] (also see chapter 1), but a causal relationship has not yet been

conclusively proven in humans. Muscle strength has not yet been evaluated in clinical studies of 11βHSD1 inhibitors. Taken together, the evidence is suggestive for upregulation of 11βHSD1 in skeletal muscle increasing the risk of muscle weakness, but larger studies with better control for potential confounders and ideally clinical trials are desirable to strengthen the evidence further.

5.3.3.2 Muscle function

Measures of muscle function and 11βHSD1 showed a degree of association, although this relationship appeared less robust than for measures of muscle strength. In the RIISC cohort, people with self-reported inability to perform usual daily activity had higher urinary (THF+5aTHF)/THE ratio. However, this association was no longer evident after adjustment for potential confounders in multivariable analysis, and urinary (THF+5aTHF)/THE ratio was not associated with self-reported ability to walk about. The dependence on indirect measures for both muscle function and 11βHSD1 activity signifies a significant limitation in the analysis of the RIISC data. In the SMK data on the other hand, muscle-specific 11βHSD1 enzymatic activity could be explored in relation to muscle function directly. Gait speed correlated negatively with muscle 11βHSD1 activity. Furthermore, there was a trend for a negative correlation between muscle 11βHSD1 activity and overall score in the Short Physical Performance Battery. The strength and direction of correlation for muscle 11βHSD1 and gait speed was similar within the CKD and healthy volunteer groups analysed separately as for the pooled analysis, albeit not statistically significant given the small sample size.

In keeping with the SMK study results, Hassan-Smith et al. previously reported weak to moderate associations for muscle 11βHSD1 mRNA expression and muscle function measurements based on jump-plate mechanometry.[193] Unfortunately, certain limitations are shared by all current studies on 11βHSD1 and muscle function, namely recruitment bias of research participants with generally good muscle function and high risk for confounding, with age being an obvious factor. Interpreting the current evidence critically, associations between 11βHSD1 levels in skeletal muscle and parameters of muscle function are suggested, but further research is needed to validate this notion. In this regard, it is important to acknowledge that physical ability is also crucially shaped by muscle-independent factors such as cognition, cardiorespiratory health or pain to name but a few.[438] Future research focusing on muscle function will do well to consider these variables in its design.

5.3.3.3 Muscle Mass

A relationship between 11βHSD1 and muscle mass is not supported by results from the SMK or RIISC cohorts. Both studies relied on indirect estimates of muscle mass. The ratio of cystatin C-based eGFR and creatinine-based eGFR has been validated previously as a surrogate marker of muscle, reflecting the proportionally larger influence of muscle mass on serum creatinine than serum cystatin C.[430, 431] Additionally, this variable had plausible associations with self-reported parameters of physical function within the RIISC dataset. Still, it must be acknowledged that a range of factors other than muscle mass can potentially influence $eGFR_{cysC}/eGFR_{creat}$ ratio. Bearing this in mind, the correlation with urinary (THF+5aTHF)/THE ratio was relatively weak and was not maintained after adjustment for potential confounders. Electrical bioimpedance analysis was employed for estimating lean tissue mass in the SMK

study. This methodology is more specific than $eGFR_{cysC}/eGFR_{creat}$ ratio and has a relevant application in the diagnosis of sarcopenia according to the EWGSOP2 definition.[14, 430] Nevertheless, lean tissue mass estimates are subject to other factors, e.g. hydration status. Accepting this limitation, neither crude nor height-adjusted appendicular muscle mass correlated with 11βHSD1 activity in quadriceps muscle biopsies.

Other studies with more accurate measurements of muscle mass had mixed findings for associations with 11βHSD1 levels in skeletal muscle tissue. Kilgour et al. found no correlation for muscle 11βHSD1 mRNA expression and muscle mass assessed by MRI.[331] A negative correlation for muscle 11βHSD1 mRNA expression and muscle mass assessed by DEXA was seen in men, but not in women in the study by Hassan-Smith et al.[193] Two clinical trials offer intriguing data on this topic among their secondary outcome measures. Administration of 11βHSD1 inhibitors led to an increase in lean tissue mass in a cohort of middle-aged overweight women and in a cohort of patients with endogenous Cushing's syndrome.[339, 340] Both studies were relatively small and unfortunately did not measure muscle strength or function parameters. Based on exploratory data from the former trial, changes in lean muscle mass may be mediated indirectly through stimulation of adrenal androgen production by 11βHSD1 inhibitors, rather than inhibition of glucocorticoid activation locally within skeletal muscle. Ultimately, 11βHSD1 activity in skeletal muscle as a determinant of muscle mass has not yet been proven for the general population at risk of sarcopenia. Any further research intending to pursue this question will do well to employ high rigour for assessing muscle mass and to corroborate any findings with measures of muscle strength and function.

In summary, evidence appears most convincing for a negative correlation between levels of 11βHSD1 in skeletal muscle and muscle strength. Based on the data presented here and reported in the literature, the relevance of 11βHSD1 in skeletal muscle as a determinant of muscle mass or composite muscle function is less clear. The majority of studies in humans to date rely on observational data, with obvious limitations for inferring a causal role. Results from clinical trials of 11βHSD1 inhibitors showing beneficial effects on lean tissue mass are intriguing. Yet, these results require validation in wider populations at risk of sarcopenia and corroboration with muscle strength and function outcomes.

5.3.4 Shifts in systemic glucocorticoid homeostasis in CKD

Glucocorticoids and their metabolites were characterised comprehensively in early morning serum samples and 24-hour urine samples in the SMK study. This provides valuable insights about shifts in glucocorticoid physiology in chronic kidney disease:

1. Early morning cortisol levels are comparable in the CKD and healthy control groups, which is consistent with many studies in the literature.[439] (see also chapter 1) It should be acknowledged however that morning serum cortisol may not accurately reflect cumulative cortisol exposure over 24 hours, as discussed in the introduction (see chapter 1). Longitudinal measurements of serum or salivary cortisol over 24 hours, which offer superior evaluation of cumulative cortisol exposure and correlate better with clinical outcomes, were not collected as part of the SMK study.
2. Serum cortisone levels are significantly reduced in the CKD group, reflecting a shift in net peripheral glucocorticoid metabolism. This is well documented in the literature,

including reduction of 11βHSD2 expression in the kidneys with declining renal function.[300, 301, 320] Whether concomitant changes in 11βHSD1 activity occur in tissues other than skeletal muscle remains unproven. As a consequence of altered peripheral glucocorticoid metabolism, the HPA axis has to adapt to maintain homeostatic regulation of serum cortisol levels. In this context, it is relevant to remember the literature that negative feedback in the HPA axis is less efficient in CKD than in health [439] (see also chapter 1), suggesting that this adaptation is incomplete.

3. Excretion of free cortisol in urine over 24 hours is reduced in CKD. Based on early morning cortisol measurements and previous studies with characterisation of diurnal cortisol profiles in CKD[271, 273], this observation is unlikely to reflect reduced circulating cortisol levels. Instead, this observation is more likely explained by reduced renal clearance of cortisol in line with reduced solute clearance in general.
4. Excretion of total glucocorticoids and their metabolites in urine over 24 hours is reduced in CKD, which is primarily due to reduced excretion of cortisone metabolites. This explains why previous studies have failed to demonstrate increasing urinary glucocorticoid excretion with declining renal function, even though diurnal profiles of serum cortisol suggest mild glucocorticoid excess. Another important consideration is that neither free cortisol nor total glucocorticoid metabolites in 24-hour urine samples are reliable tests for the diagnosis of suspected endogenous Cushing's syndrome in people with impaired kidney function, as both measures can be falsely low. The most appropriate alternative test may be measurements of free cortisol in saliva.
5. The correlation between urinary (THF+5aTHF)/THE ratio and 11βHSD1 enzymatic activity in skeletal muscle was weak in a cohort with mixed kidney function (i.e. pooled

analysis of the CKD and healthy groups). In other words, urinary (THF+5aTHF)/THE ratio has little specificity as a marker of muscle 11βHSD1 activity. This observation does not necessarily contradict previous validations that urinary (THF+5aTHF)/THE ratio is sensitive to systemic changes in 11βHSD1 activity in populations with normal kidney function. Yet, it highlights that results based on urinary (THF+5aTHF)/THE ratio require cautious interpretation. This is true for analysis of the RIISC dataset and may explain certain discrepancies to findings in the SMK study.

5.3.5 Summary

In conclusion, measurement of 11βHSD1 activity in skeletal muscle biopsies revealed no difference for people with and without CKD. These measurements did however validate correlations for biological activity of 11βHSD1 in skeletal muscle with age and muscle strength, which had previously only been described for mRNA expression of 11βHSD1. Furthermore, detailed characterisation of glucocorticoids in the SMK study has confirmed and refined notions of altered glucocorticoid physiology in CKD. Lastly, interpretation of the data from the RIISC cohort has proven challenging, reflecting limitations of surrogate markers in the absence of direct measurements of 11βHSD1 or muscle phenotype.

Chapter VI - General Discussion

6.1 Regulation of 11βHSD1 in skeletal muscle in CKD

This PhD thesis describes a clinical case control-study, a mouse model and human cell culture experiments to address the question whether excess sarcopenia risk in chronic kidney disease is attributable to overactive glucocorticoid activation by 11βHSD1 in skeletal muscle. Direct ex vivo measurement in fresh skeletal muscle biopsies showed similar 11βHSD1 enzymatic activity for patients with pre-dialysis chronic kidney disease and healthy age-matched volunteers. Data from preclinical models corresponds with these clinical study results. Mice with adenine-induced persistent renal impairment exhibited no upregulation of 11βHSD1 mRNA expression in skeletal muscle or elevated 11βHSD1 activity ex vivo. Furthermore, serum from people with CKD did not induce upregulation of 11βHSD1 mRNA expression or enzymatic activity in human primary muscle cell cultures compared to serum from age-matched donors without kidney disease. Therefore, evidence from independent lines of investigation supports the null hypothesis that persistent reduction in kidney function alone does not substantially change 11βHSD1 function in skeletal muscle tissue.

This report describes the first direct quantification of 11βHSD type 1 function in humans with CKD. Previous studies have shown shifts in systemic ratios of active and inactive glucocorticoid metabolites that suggest increased global 11βHSD1 activity in CKD.[300, 320] Further supportive findings from preclinical models [259, 324] have led to the hypothesis that 11βHSD1 activity is elevated in CKD. However, the situation in CKD is complicated by changes in 11βHSD type 2-mediated glucocorticoid inactivation, as evidenced by reduced expression of 11βHSD type 2 mRNA and protein in renal biopsies.[301] Concomitant changes in 11βHSD2 activity can also influence the profile of glucocorticoid metabolites in urine. Therefore, in the

absence of direct measures of 11βHSD1, some caution is required in the interpretation of previous studies. Certainly, data from the SMK study in this thesis illustrates that urinary ratios of active and inactive glucocorticoids do not accurately correlate with 11βHSD1 activity measured in skeletal muscle biopsies in cohorts with mixed kidney function.

In the clinical study, direct quantification of 11βHSD1 function was performed only for skeletal muscle tissue. This is arguably the most relevant site of activity in relation to skeletal muscle health and sarcopenia risk. Yet, liver and fat are also major sites of 11βHSD1 activity with a major proportional contribution to global 11βHSD1 activity in the body.[189, 190] Previous reports indicated that uraemic conditions upregulate 11βHSD1 in liver and potentially also in fat.[259, 324] In the mouse model reported here, hepatic and adipose 11βHSD1 enzymatic activities ex vivo were not significantly different for animals with and without renal impairment. The discrepancy to previous results may reflect differences in implementation of the animal model in terms of adenine dosage and duration. Given these preclinical findings and lack of direct human data, there remains an unresolved question whether 11βHSD1 function is altered in tissues other than skeletal muscle in human CKD. Answering this question is not trivial. Concurrent changes in 11βHSD2 activity interfere with non-invasive assessments of hepatic 11βHSD1 function (e.g. prednisone challenge test) and risks related to liver biopsy represent an ethical hurdle to obtaining hepatic tissue. Pharmacokinetic studies with radiolabelled glucocorticoid tracers may represent a feasible strategy for future research.[190]

Inflammation is a known potent stimulus for upregulation of 11βHSD1 in skeletal muscle and other tissues.[182, 191, 263] This is evidenced in the cell culture experiments when human

muscle cells were treated with TNFα. Despite clear evidence for renal inflammation in adenine-fed mice, no change was observed for inflammatory markers in muscle tissue. Inflammation in the adenine model can be more pronounced when more severe regimes of adenine are used.[401, 402] If inflammation is the predominant – and potentially singular – regulator of 11βHSD1 function in renal impairment, lack of significant systemic and intra-muscular inflammation can explain absent change in 11βHSD1 activity in the animal model. In line with this assumption, the animal experiment and the cell culture experiment failed to identify alternative regulators of 11βHSD1 in uraemia besides inflammation.

Chronic kidney disease is characteristically accompanied by elevation in inflammatory cytokines.[55, 138, 440, 441] This has been established in multiple observational studies. Yet, differences in circulating cytokine concentrations are often very small for a majority of patients with CKD compared to the general population, especially among ambulatory patients without end-stage renal failure. Inflammatory phenotyping for SMK study participants is outstanding and will be informative for interpreting the findings on skeletal muscle 11βHSD1 activity. Many studies that have reported upregulation of 11βHSD1 in other conditions have investigated cohorts with acute flares of inflammation, rather than indolent chronic disease.[321, 323, 351] This raises the question whether low grade or chronic elevation of circulating cytokines is a sufficient stimulus for inducing changes in 11βHSD1 activity, or whether 11βHSD1 regulation is more sensitive to flares of acute inflammation. Besides low-level elevation of inflammatory cytokines, patients with CKD are more susceptible to intercurrent infections and cardiovascular events.[442, 443] Accordingly, flares of acute inflammation occur more frequently in this patient population than in the general population, especially in patients with multiple

comorbidities or patients receiving renal replacement therapy. This may cause transient upregulation of 11βHSD1. However, given the sample size and selection criteria for the SMK study, the study had limited ability to detect transient 11βHSD1 overactivity in CKD or identify 11βHSD1 overactivity in specific subgroups within the CKD population. Considering the technical challenges and participant burden associated with accurate determination of 11βHSD1 activity in people with CKD, any future research efforts in this direction will need to pay particular attention to participant selection criteria.

A notable finding in the clinical study was the association of muscle 11βHSD1 activity with age. This result was not fully expected, given the sample size and age restriction for participant recruitment (60-80 years). Still, this observation fits with the wider body of literature. It is the first demonstration of 11βHSD1 enzymatic activity in skeletal muscle correlating positively with age, but similar observations have been reported for 11βHSD1 mRNA expression.[193, 331] Furthermore, the relationship between 11βHSD1 regulation and age appears conserved across multiple tissues.[434-437] CKD, like other chronic diseases, has been perceived as a condition of accelerated aging.[444, 445] It is intriguing to speculate whether this may bring about an interaction between age and CKD status for regulation of 11βHSD1. A robust examination of this proposition lies outside the scientific body of work presented here and may inform future research.

6.2 The role of 11βHSD1 in skeletal muscle for the pathophysiology of sarcopenia

Ample evidence supports the direct myopathic effects of excess glucocorticoid signalling in skeletal muscle. This has been reviewed in section 1.6.1 and shown again in the cell culture experiment, when cortisol induced dose-dependent reduction in protein synthesis and increase in protein degradation in human muscle cells. The significance of 11βHSD1 for the pathophysiology of sarcopenia is more challenging to elucidate. Conceptually, local amplification of glucocorticoid signalling within skeletal muscle tissue represents a plausible mechanism for raised sarcopenia risk. Indeed, the SMK study identified negative correlations between quadriceps muscle 11βHSD1 activity and muscle strength (grip strength) and muscle function (gait speed), despite a relatively small sample size. This corroborates previous observations, which have only shown this pattern for 11βHSD1 mRNA expression rather than biological activity. [193, 331] Scrutinizing this finding further, regression modelling reveals that muscle 11βHSD1 activity correlates more closely with grip strength than participants' age in the SMK cohort, and that the relationship is independent of participants' gender. These results comply with predictions from the hypothesis that glucocorticoid activation by 11βHSD1 within skeletal muscle contributes to development of sarcopenia. Importantly however, this type of observational data could still be affected by confounders, meaning it does not prove a causal relationship.

Further inferences about causality and disease mechanisms may be gleaned from the preclinical models. In the conduct of an animal experiment, interventions to manipulate 11βHSD1 function are possible in ways that are difficult or impossible in clinical studies.

Genetic deletion of 11βHSD1 had no impact on the manifestation of muscle atrophy in the implemented mouse model of persistent renal impairment. This observation argues against the hypothesis that 11βHSD1 activity contributes to muscle loss in CKD in a meaningful fashion. The fact that 11βHSD1 activity was not significantly different in skeletal muscle of animals with renal impairment corresponds with observations from the clinical study, hence strengthening the validity of the model. In the end, the applicability of animal research to human disease is always hampered by species differences. Notwithstanding, these findings represent the best available evidence to address the underlying mechanistic aspects of the research hypothesis.

It remains unknown whether the muscle phenotype would be different, if the animal experiment were to be repeated with muscle-specific rather than global suppression of 11βHSD1 activity. The animal model highlighted compensatory responses that occur with global suppression of 11βHSD1 function, namely upregulation of adrenal-derived glucocorticoid. Previous attempts to generate a genetically altered mouse with muscle-specific 11βHSD1 genetic knock-out have been unsuccessful, with persistence of enzymatic activity in muscle despite marked suppression of mRNA expression.[446] Alternative experimental strategies exist and have not yet been attempted, for example intramuscular delivery of gene knock-down agents or tissue-selective overexpression of 11βHSD2. Those avenues of research were not pursued in light of limited research resources and negative results with the global 11βHSD1 knock-out mouse.

This PhD thesis focussed on the role of 11βHSD1 for loss of muscle mass and strength within the narrower remit of chronic kidney disease. The findings do not necessarily exclude a role of 11βHSD1 for muscle deterioration in other clinical settings. Other mouse studies found that genetic deletion of 11βHSD1 protects against muscle atrophy in exogenous glucocorticoid excess, exacerbates muscle atrophy in inflammatory arthropathy models, and has no impact on muscle atrophy in aged mice.[263, 329, 403] These diverging results emphasize how 11βHSD1 can have dichotomous effects on muscle phenotype. It is a reminder of the equipoise that existed around the original research hypothesis that 11βHSD1 contributes to sarcopenia in CKD, when the state of CKD combines a multitude of pathologies including immunological and endocrine changes. It also opens the possibility that 11βHSD1 activity in skeletal muscle could exert pro-sarcopenic or anti-sarcopenic effects in certain subgroups of patients with CKD. This remains speculative, pending further clinical research.

Open questions remain about the role of glucocorticoid metabolism and signalling more widely for sarcopenia in CKD. The current literature on glucocorticoid signalling effects on muscle deterioration in CKD has been summarised in section 1.6, followed by a review of changes to glucocorticoid balance in CKD in section 1.7. Preclinical models have suggested that intramuscular glucocorticoid signalling is an obligatory cofactor for muscle loss in the setting of acidosis and impaired insulin signalling.[217, 257] However, clinical evidence to corroborate the significance of glucocorticoid signalling for sarcopenia in human CKD remains scarce (reviewed in section 1.6.2). A range of glucocorticoid indices in serum and urine, which were determined as part of the SMK study, did not produce superior associations with muscle phenotype than muscle 11βHSD1 activity. As a caveat, these data have an exploratory

character in view of the sample size and inherent design of the SMK study. A need to tackle anabolic resistance in CKD and sarcopenia warrants further research into the role of glucocorticoids. It is crucial that such research is mindful of the challenges surrounding accurate assessment of glucocorticoid status in CKD, where abnormalities are subtle and can be masked by altered renal metabolism and excretion.(reviewed in section 1.7) Diurnal profiles of salivary cortisol would offer a meaningful and convenient measure.[214, 297] Another strategy lies with more detailed exploration of intramuscular glucocorticoid signalling in biopsies from patients with CKD. Using RNA, protein or histology-based biomolecular techniques, it may be possible to glean additional insights about the glucocorticoid signal transduction machinery and signalling status from preserved muscle tissue samples from the SMK study.

6.3 Therapeutic efficacy of 11βHSD1 inhibition to mitigate muscle atrophy in renal impairment

A principal aim of this research project is evaluation of 11βHSD1 inhibition as a therapeutic strategy against sarcopenia in CKD. While the cell culture and clinical data offer important contextual information, the animal experiment addresses this question in the most direct fashion. Other studies have shown that suppression of 11βHSD1 activity can have diverging and even diametrically opposed effects on muscle phenotype depending on biological context. [263, 329, 403] In the mouse model of adenine-induced renal impairment studied here, genetic elimination of 11βHSD1 function did not reduce measures of muscle atrophy. The experimental premises of establishing a tolerable model of persistent renal impairment with accompanying muscle atrophy and supressing enzymatic activation of glucocorticoid in

peripheral tissues were met, giving legitimacy to the findings. Residual uncertainty about the chief drivers of muscle atrophy in the model hamper mechanistic interpretation, but this does not undermine the central findings. Factors that may have thwarted therapeutic success include absence of marked systemic glucocorticoid excess with renal impairment, absence of elevated 11βHSD1 activity in skeletal muscle with renal impairment and compensatory upregulation of adrenal glucocorticoid production with 11βHSD1 blockade. All three considerations can be assumed to apply in human CKD too, based on findings in this PhD thesis and evidence from the literature.[333, 429] Hence, the knowledge generated in the process of this PhD thesis reduces the likelihood that inhibition of 11βHSD1 represents an effective therapy to mitigate the excess sarcopenia risk that is attributable to chronic kidney disease.

As a concluding thought, it is worth considering if and where a therapeutic niche for 11βHSD1 inhibitors to treat sarcopenia may exist besides CKD. Based on our discussion so far, this is most likely to be the case when the following conditions apply. Firstly, glucocorticoid-dependent pathways should have a significant contribution to the myopathy. Secondly, peripheral glucocorticoid activation and local amplification of glucocorticoid signalling should have a meaningful impact on intramuscular glucocorticoid signalling, beyond homeostatically maintained systemic glucocorticoid levels. Thirdly, inhibition of 11βHSD1 should be implemented in a manner that avoids compensatory HPA axis upregulation. When HPA axis upregulation is unavoidable, a differential rise in adrenal androgens relative to circulating glucocorticoids may still confer a benefit on skeletal muscle.[340] The final paragraphs will explore these considerations for inflammatory disorders, aging, glucocorticoid excess and

muscle-specific targeting of 11βHSD1 inhibition to broaden perspectives on future research into 11βHSD1 inhibitors.

6.4 Future perspectives on 11βHSD1 inhibition as a strategy to counter sarcopenia in other settings

Systemic inflammatory disorders encompass a higher risk for sarcopenia, regardless of autoimmune, infective or other aetiology.[139, 260] Both systemic glucocorticoid production and peripheral glucocorticoid activation are elevated with inflammation, including 11βHSD1 activity in skeletal muscle.[310, 351] 11βHSD1 inhibition has therefore been examined in previous studies as a therapeutic strategy to counter muscle atrophy, albeit only in animal models and not in clinical trials. Loss of 11βHSD1 function produced detrimental effects on muscle phenotype in two inflammatory mouse models (transgenic TNFα expression modelling inflammatory arthropathy and intra-tracheal instillation of lipopolysaccharide modelling acute exacerbation of chronic obstructive pulmonary disorder).[263, 404] These observations serve as a reminder of the glucocorticoid see-saw of beneficial anti-inflammatory effects and harmful myopathic effects. When 11βHSD1 inhibitors are applied for treatment indications with systemic inflammation, the risks of exacerbating inflammation-driven pathology could outweigh potential benefits from counteracting myopathy attributable to endogenous glucocorticoid signalling. This limitation may fundamentally limit the usefulness of systemic 11βHSD1 inhibition in conditions with a significant inflammatory component, unless concurrent inflammation is controlled by concurrent therapies.

Age-related sarcopenia could be contemplated as another potential indication for 11βHSD1 inhibitors. Age is one of the strongest risk factors for sarcopenia.[7] Furthermore, upregulation of 11βHSD1 in skeletal muscle with age has been evident in this PhD thesis and other studies.[193, 331] The safety profile of pharmaceutical 11βHSD1 inhibitors has been acceptable in Phase II trials with aging populations, when these were tested for indications of osteoporosis or dementia.[337, 447] Yet, the relevance of 11βHSD1 for the pathophysiology of sarcopenia in the general population remains uncertain, as current evidence in humans is only observational and susceptible to confounding. Regrettably, previous clinical trials of 11βHSD1 inhibitors in aging populations have not reported muscle-related outcome measures. The best available evidence again comes from an animal experiment. Aged male mice with germline genetic deletion of 11βHSD1 had no better grip strength or greater quadriceps muscle weight than aged wild-type control animals.[403] It worth pointing out that serum corticosterone levels were elevated in young and old 11βHSD1-KO animals, meaning those animals were exposed to systemic glucocorticoid excess throughout their lifetime. This does not reflect how 11βHSD1 inhibition would be applied in humans. In another deviation from human translation, aged mice exhibited no upregulation of 11βHSD1 in skeletal muscle or other tissues. Despite these limitations, the preclinical results evoke doubts about the viability of 11βHSD1 inhibitors to treat age-related sarcopenia.

Glucocorticoid excess arguably presents the most promising indication for 11βHSD1 inhibitors to limit sarcopenia. The direct role of glucocorticoid signalling in muscle to cause myopathy is undisputed in endogenous as well as exogenous glucocorticoid excess.(reviewed in section 1.6.1) In mouse models of exogenous glucocorticoid excess, genetic deletion of 11βHSD1

prevents myopathy.[329, 351] Additionally, there is limited clinical data that suppression of 11βHSD1 function is protective for lean body mass in the context of endogenous glucocorticoid excess. Such observations derive from a case report with mutation in the 11βHSD1 gene and a non-controlled clinical study of 11βHSD1 inhibitor.[332, 339] A critical consideration for the postulated efficacy of 11βHSD1 inhibition in glucocorticoid excess lies with the elimination of compensatory HPA axis regulation, either through loss of feedback mechanisms in endogenous glucocorticoid excess or through adrenal suppression with exogenous glucocorticoids. Furthermore, glucocorticoid signalling itself induces 11βHSD1 activity, leading to an amplification cycle that can be targeted by 11βHSD1 inhibition.[192] The most rigorous study of 11βHSD1 inhibition in glucocorticoid excess to date has been conducted by Othonos et al.[448] In an experimental medicine study of 11βHSD1 inhibitor to mitigate prednisolone-induced adverse effects, the 11βHSD1 inhibitor AZD4017 had beneficial effects on glucose metabolism, lipid metabolism, bone turnover and blood pressure compared to the placebo control. Further data on skeletal muscle markers from this study is awaited with interest, as well as results from ongoing studies of the 11βHSD1 inhibitor SPI-62 in cohorts with endogenous or therapeutic glucocorticoid excess.(clinicaltrials.gov: NCT05436639, NCT05307328, NCT05436652)

As a final thought, it is worth considering tissue-selective targeting of 11βHSD1 in skeletal muscle as a potential therapeutic strategy to alleviate sarcopenia. Preclinical studies have clearly shown that glucocorticoid-induced myopathy is dependent on intramuscular glucocorticoid signal transduction.[217, 238] Dampening intramuscular glucocorticoid signalling through tissue-selective 11βHSD1 suppression may offer a strategy to prevent

muscle loss and weakness, while maintaining physiological circulating glucocorticoid levels and avoiding compensatory HPA axis upregulation. So far, this principle has only been tested successfully in vitro and not yet in vivo.[326] Two fundamental questions persist with this approach; whether 11βHSD1 is indeed the optimal pharmacological target and how muscle-selective pharmacological targeting can be achieved.

Firstly, targeting 11βHSD1 within skeletal muscle can never achieve more than partial inhibition of glucocorticoid signalling, abolishing activation of inactive glucocorticoid but inert to circulating active glucocorticoid. In contrast, muscle-specific glucocorticoid receptor antagonism or creation of a glucocorticoid-free niche through 11βHSD2 overexpression offer greater capacity for supressing intramuscular glucocorticoid signalling. Potent suppression of intramuscular glucocorticoid signalling could enhance therapeutic efficacy against sarcopenia, or it could potentially upset physiological roles of glucocorticoids to maintain healthy muscle turnover and function.[183] The application of anti-glucocorticoid strategies to mitigate sarcopenia risk represents an interesting area for further research.[239]

Secondly, tissue-specific targeting of therapeutics to skeletal muscle represents a prevailing technical challenge. A range of biomolecular and biomedical strategies are in development for muscle-specific delivery of therapeutics, including linkage of therapeutics to ligands for cell-surface receptors, engineered viral vectors to facilitate transfection, optimisation of oligonucleotide therapeutics for preferential uptake, and insertion of genetically altered progenitor cells into the myocellular pool. [221, 449-452] These efforts have yielded some encouraging results, with several technologies in clinical trials and an oligonucleotide-based

therapy licenced for treatment of muscular dystrophy.[453, 454] Such novel technologies could have a profound impact on future research to develop treatments for sarcopenia.

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Appendix

8.1 Appendix I: Gene probes used in real-time PCR to measure mRNA expression

Target gene	Species	Taqman™ Gene Expression Assay ID
18S	mouse	n/a – supplied by Applied Biosystems™
GAPDH	mouse	Mm99999915_g1
HSD11B1	mouse	Mm00476182_m1
GILZ (TSC22d2)	mouse	Mm00726417_s1
H6PD	mouse	Mm00557617_m1
FOXO1	mouse	Mm00490671_m1
TRIM63	mouse	Mm01185221_m1
FBXO32	mouse	Mm00499523_m1
HSD11B2	mouse	Mm01251104_m1
MSTN	mouse	Mm01254559_m1
IGF1	mouse	Mm00439560_m1
IL6	mouse	Mm00446190_m1
TNF	mouse	Mm00443258_m1
IL1B	mouse	Mm00434228_m1
GAPDH	human	Hs99999905_m1
MYF5	human	Hs00929416_g1
MYF6	human	Hs01547104_g1
MYOD	human	Hs02330075_g1
MYOG	human	Hs01072232_m1
HSD11B1	human	Hs01547870_m1
GILZ (TSC22d2)	human	Hs00608272_m1
H6PD	human	Hs00188728_m1
IL6	human	Hs00174131_m1
IL1B	human	Hs01555410_m1
TNF	human	Hs00174128_m1
FOXO1	human	Hs01054576_m1
FBXO32	human	Hs01041408_m1
TRIM63	human	Hs00261590_m1
IGF1	human	Hs01547656_m1
IGF2	human	Hs03929076_gH
IRS1	human	Hs00178563_m1
MSTN	human	Hs00976237_m1

8.2 Appendix II: Detailed methods for quantification of steroids in human samples

The methodology for urine steroid analysis has previously been described in (Sagmeister et al., 2019) and is reproduced here with minor adaptations:

Glucocorticoids and their metabolites were quantified in urine by liquid chromatography – tandem mass spectrometry. This protocol has previously been described in detail and validated. (Bancos et al., 2020; Sagmeister et al., 2019) Steroids were extracted from 200µL of spot urine samples (RIISC study) or 24-hour urine samples (SMK study). Urine samples were spiked with 0.4µg of deuterium-labelled internal standard (THE-d5, THF-d5, cortisone-d (Isosciences, Ambler, PA, USA) and cortisol-d4 (Sigma Aldrich, UK)). Steroids were subsequently hydrolysed to remove their conjugate groups (sulphate and/or glucuronide). This was achieved by heating the urine for 3 hours at 60^{o}C in a hydrolysis buffer (400µL of 0.2mol/L acetate buffer pH 4.8-5: with 10mg sulphatase [adjusted based on batch activity] and 10mg ascorbate/3mL of solvent). The now "free" deconjugated steroids were subsequently extracted by solid phase extraction using 96-well C_{18} 100mg cartridges in a positive pressure manifold, eluting with 800µL of methanol. The samples were dried at 50^{o}C under nitrogen and reconstituted in 200µL of 50/50 methanol/water for analysis via liquid chromatography–tandem mass spectrometry (uPLC-MS/MS).

A Waters Xevo-XS mass spectrometer coupled to an Acquity uPLC with an electrospray ionization source in positive ionization mode was utilized in these experiments. Cortisol, cortisone, THE, THF and 5αTHF were separated on a BEH C18 1.7µm 5cm column at 60^{o}C with the mobile phases methanol and water both with 0.1% formic acid. Gradient profile started at 30% methanol, was held for one minute and then moved with a linear gradient to 60% methanol at 5 minutes, followed by washing and re-equilibration steps. Steroids were identified by comparison to authentic reference standards, with a matching retention time and identical mass transitions (MRMs) required for positive identification. Steroids were quantified relative to a calibration series ranging from 0.5-5000 ng/mL prepared in phosphate buffered saline with 0.1% bovine serum albumin, including a blank. Steroid concentrations were calculated relative to their assigned internal standard.

The methodology for serum steroid analysis has previously been described in (Hardy et al., 2021) and is reproduced here with minor adaptations:

Serum steroids were analysed using liquid chromatography–tandem mass spectrometry (LC-MS/MS), as previously described. (Hardy et al., 2021; O'Reilly et al., 2017; O'Reilly et al., 2019; Lina Schiffer et al., 2023; L. Schiffer et al., 2022) An internal standard mixture was added to 200µL of serum. Steroids were extracted via liquid/liquid extraction with 1mL of tert-butyl methyl ether (MTBE). The MTBE layer was removed, evaporated to dryness, and reconstituted

in methanol/water prior to LC-MS/MS analysis. The extracts were analysed on a Xevo-XS triple quadrupole mass spectrometer (Waters) coupled to an Acquity ultra high-performance liquid chromatography system (UPLC) (Waters). Steroids were separated on a HSS T3 (1.8µm) column (Waters) using a methanol/water (0.1% formic acid) gradient with post column infusion at 5µL/min of 6mM ammonium fluoride. Starting condition was 45% methanol, held for 1 minute, followed by a linear gradient to 75% methanol at 5 minutes. Subsequently, the column was washed at 98% methanol and reconditioned at starting condition prior to the next injection. Steroids were identified and quantified via comparison to reference standards; positive identification was confirmed via matching retention time and two identical mass transitions. The steroids quantified in this method were cortisol and cortisone. All steroids were quantified based on a calibration series ranging from 0.01 to 250 ng/mL (including a blank; 0 ng/mL calibrator).

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