The role of pre-receptor glucocorticoid metabolism in chronic inflammatory disease-associated muscle atrophy

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Key words: Muscle atrophy, glucocorticoids, inflammation, 11beta hydroxysteroid dehydrogenase 1

The role of pre-receptor glucocorticoid metabolism in chronic inflammatory disease-associated muscle atrophy

by

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Abstract

Chronic inflammatory diseases (CIDs) such as rheumatoid arthritis (RA) and chronic obstructive pulmonary disease (COPD) are associated with prolonged periods of local and systemic inflammation. As a result, patients with CIDs often encounter co-morbidities such as osteoporosis and skeletal muscle wasting. Glucocorticoids (GCs) are steroid hormones widely used in the treatment of CIDs due to their potent anti-inflammatory effects. Despite their efficacy, their clinical application is limited due to undesirable side effects including osteoporosis and muscle wasting. The enzyme 11 beta-hydroxysteroid dehydrogenase 1 (11β-HSD1) is found in abundance in tissues such as skeletal muscle and bone and converts inactive GCs to their active counterparts, increasing local GC activity. In this thesis we look at the contribution of 11β-HSD1 in mediating GC-induced muscle wasting in RA and acute exacerbations of COPD (AE-COPD). Using mouse models of RA and AE-COPD, we look at the impact global genetic deletion of 11β-HSD1 has in muscle and bone loss with inflammation or GC-excess. In emphysematous animals with pulmonary inflammation, genetic deletion of 11β-HSD1 resulted in exacerbated muscle atrophy in addition to increased corticosterone levels compared to wild-type (WT) controls. Mice with genetic overexpression of human TNF-α (TNF-tg) receiving corticosterone demonstrated a marked increase in muscle wasting compared to untreated mice. In contrast, a partial protection against muscle wasting was observed in 11β-HSD1 null

mice with GC-treated polyarthritis. Taken together, our data suggest 11β-HSD1 inhibition in AE-COPD and RA may not protect against endogenous GC-driven muscle wasting, however when combined with therapeutic GCs, 11β-HSD1 inhibition may offer protection against muscle wasting in such disease settings.

Table of Contents

Chapter 1 - General Introduction ____ 1

Chapter 2 - Inflammation and Skeletal Muscle Wasting During Cachexia ____ 42

Chapter 3 - 11β-HSD1 determines the extent of muscle atrophy in a model of acute exacerbation of COPD ____ 112

Chapter 4 - Exploring the Interface between Inflammatory and Therapeutic Glucocorticoid Induced Bone and Muscle Loss ____ 153

Chapter 5 – Therapeutic glucocorticoids prevent bone loss but drive muscle wasting when administered in chronic polyarthritis ____ 197

Chapter 6 – Global deletion of 11β-HSD1 prevents muscle wasting associated with glucocorticoid therapy in polyarthritis ____ 234

Chapter 7 – Summary and General Discussion ____ 271

Abbreviations ____ 304

Acknowledgements ____ 313

***Curriculum Vitae* ____ 317**

Chapter 1 - General Introduction

Chronic inflammatory disease (CID) encompasses a multitude of diseases such as rheumatoid arthritis (RA) and chronic obstructive pulmonary disease (COPD) and often involve extended periods of local and systemic inflammation. As a consequence of chronic inflammation, patients with CID often experience co-morbidities such as osteoporosis and muscle wasting, leading to a poorer quality of life and prognostic outcome. Glucocorticoids (GCs) are steroid hormones that are potent anti-inflammatories and are widely used in the treatment of CIDs, however, their long-term application is associated with undesirable side effects including muscle wasting [1]. The enzyme 11 beta-hydroxysteroid dehydrogenase 1 (11β-HSD1) primarily converts inactive GCs to their active counterparts (cortisone and 11-dehydrocorticosterone to cortisol and corticosterone respectively) [2], and as a result increases local active GCs within tissue. Therefore, the general introduction in Chapter 1 introduces muscle physiology (both healthy and non-healthy), chronic inflammatory disease, GCs, and pre-receptor metabolism of GCs.

1.1 Skeletal Muscle in health and disease

Skeletal muscle is one of the largest tissues of the body, making up 40% of total body mass [3]. Its main functions are to provide locomotion and support to the skeleton and body; however, it also plays a crucial role in energy metabolism and glucose homeostasis [3].

1.1.1 Embryogenesis

During embryogenesis, skeletal muscle is formed from segmented mesodermal cells which divide along the embryo, forming somites [4]. There are three sections of the somite, known as the sclerotome, dermatome, and the myotome. The dorsal part of the somite, known as the dermomyotome, then divide further forming the epimere and the hypomere which form epaxial and hypaxial muscles in response to neural signals [5]. Muscle progenitor cells known as myoblasts then either remain in the somite or migrate to the limbs and trunk and around the body [5, 6], controlled by myogenic transcription factors such as Paired box protein 3 (Pax3) and 7 (Pax7). Upon expression of myogenic regulatory factors (MRFs); myoblast determination protein 1 (MyoD) and myogenin (MyoG) [7, 8], myoblasts undergo skeletal muscle differentiation and eventually fuse together to form single multinucleated cells referred to as myotubes. In the latter stages of embryonic myogenesis, undifferentiated somite-derived precursor cells known as satellite cells (SCs) remain in the basal lamina of myotubes, which are essential to growth and regeneration of adult skeletal muscle [9].

1.1.2 Basic muscle physiology

Skeletal muscle is comprised of numerous multinucleated muscle cells, known as myofibers [10]. Within these myofibers are myofibrils arranged in bundles and surrounded by the cell membrane known as the sarcolemma [10]. These

myofibrils consist of sarcomeres which are predominantly formed by organised actin and myosin proteins, forming thin and thick filaments respectively, which overlap to form cross-bridges during contraction (Figure 1) [10].

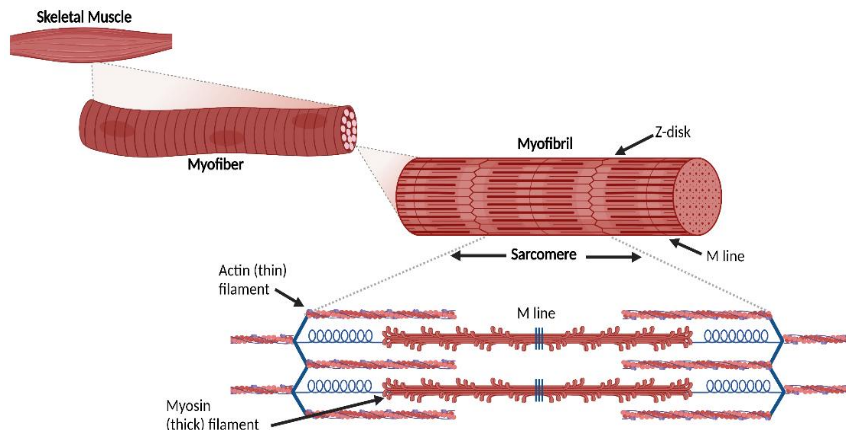

Figure 1. Structure of skeletal muscle. Contractile proteins are organised intro myofibrils. Myofibrils form bundles that make up myofibers, of which skeletal muscle is comprised of. Created with BioRender.

Following action potential stimulation, ATP binds to the myosin head leading to its dissociation from actin [11]. Myosin then binds to actin at a different location along the filament as a result of ATP hydrolysis, leading to the myosin filament sliding longitudinally along the actin filament [11].

In skeletal muscle myofibers are made up of a variety of distinct fiber types that endow unique properties with different ratios within the whole muscle and are responsive to external stimuli such as exercise, hormones, oxygen levels and nutrients [12]. There are two main types of fibers found in skeletal muscle, Type I

(slow-twitch) and Type II (fast-twitch), of which have varying expressions of isoforms of myosin heavy chain proteins (Table 1). Type I fibers have a relatively high oxidative capacity and are characterized by their resistance in fatigue of muscle [13]. Type II fibers have three isoforms: type IIa, IIb and IIx, of which all have a relative high capacity to use glycolysis to generate energy for contraction, however they are prone to fatigue more quickly [13].

Fiber Type	Myosin Heavy Chain	Contraction Speed	Activity	Glycolytic Capacity	Oxidative Capacity
Type I	MYH7	Slow	Aerobic	Low	High
Type IIa	MYH2	Relatively Fast	Long anaerobic	High	High
Type IIx	MYH1	Fast	Short anaerobic	High	Intermediate
Type IIb	MYH4	Very Fast	Short anaerobic	High	Low

Table 1. Summary of muscle fiber types.

Evidence shows there is a correlation between muscle fiber size, often determined by cross sectional area (CSA), muscle mass, and strength. During times of inactivity or muscle atrophy, CSA of fibers are reduced, rather than a reduction in quantity of fibers [14]. This reduction is heavily associated with a loss of skeletal muscle strength, affecting physical functions such as walking [15]. Changes observed in muscle mass and fiber size are attributed to several processes, including proteolytic and anabolic metabolism of muscle proteins, and myonuclear turnover.

1.1.3 Muscle mass regulation during homeostasis

Muscle mass is determined by the dynamic balance between muscle growth and repair, and muscle degradation and wasting, in which several processes are involved including muscle protein turnover (synthesis and degradation) and myonuclear turnover (myonuclear accretion and degradation). Changes in these processes are seen in response to injury, repair, disease and aging, and under healthy conditions these processes are balanced when muscle mass homeostasis is restored.

Myonuclear turnover

Myonuclear turnover plays an important role in determining muscle mass, muscle regeneration and recovery, and is determined by the balance between myonuclear accretion and apoptosis [16]. Satellite cells, the precursors cells of muscle, remain in a state of quiescence in undamaged muscle cells due to their expression of Pax7, a mediator that inhibits myogenesis [7]. Found between the basal lamina and sarcolemma of myofibers, dormant heterogeneous SC populations rapidly activate and proliferate following exercise and injury [17]. Upon activation, SCs migrate to the site of damage and downregulate Pax7, which in turn increases other proliferation and differentiation factors such as MyoG, MyoD and Myf5. This increased expression of myogenic factors promotes fusion of myoblasts to existing or new muscle fibers [18] (Figure 2). Myostatin, also known as growth differentiation factor 8 (GDF8) is a growth differentiation factor and a member of the TGF-β family

[19]. Produced and secreted by myocytes, myostatin acts as a negative regulator of muscle mass through its capacity to inhibit myogenesis upon its binding to the activin type IIB receptors (ActRIIB) (Figure 2) [20]. Positive regulators of myogenesis, such as insulin-growth factor 1 (IGF-1), have been shown to be important in maintenance of skeletal muscle. Increased levels of IGF-1 following exercise or muscle injury increases proliferation capacity of SCs and stimulates myogenic differentiation and fusion (Figure 2) [21]. More detailed descriptions of myogenesis and apoptosis in skeletal muscle are discussed further in Chapter 2 (page 52) and Chapter 4 (page 168).

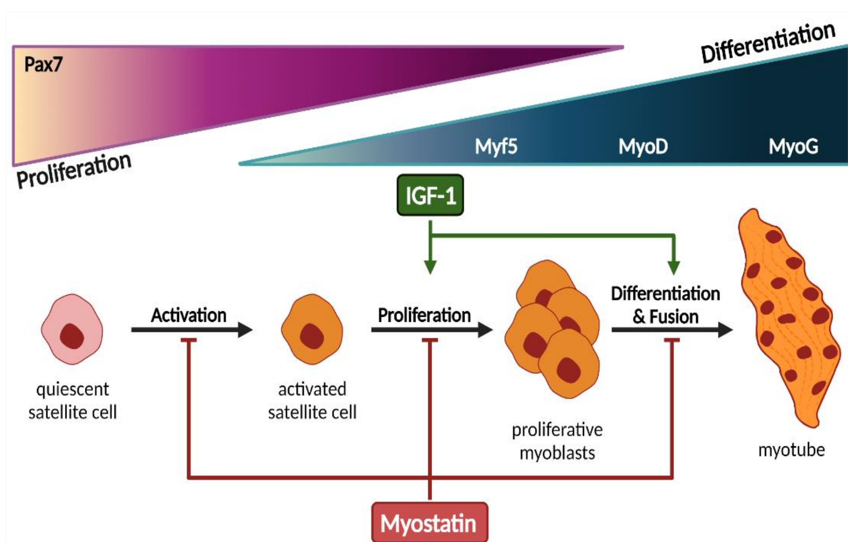

Figure 2. Schematic overview of myonuclear accretion. Signalling molecules involved in proliferation and differentiation and change in expression levels. Myostatin is able to inhibit myogenesis, whilst IGF-1 is able to stimulate myogenesis. Created with BioRender.

Protein turnover

Maintenance and changes in muscle mass are largely dependent on protein turnover, which is defined as the balance between muscle protein synthesis and degradation. These cellular processes are explained in further detail in Chapter 2 (page 49) and Chapter 4 (page 168). In brief, elevated protein synthesis requires increased mRNA translation and can be induced by activation of the Akt/mTOR pathway via Akt phosphorylation as a result of IGF-1 binding (Figure 3) [22]. Upon IGF-1 binding, phosphoinositide 3-kinase (PI-3K) activates and leads to the phosphorylation of Akt, and its subsequent activation. Phosphorylated Akt then activates mTOR complex 1 (mTORC1), with TSC2 deactivation lifting mTOR inhibition [23]. The activation of mTOR initiates mRNA translation through dissociation of inhibiting eIF4E from a phosphorylated 4EBP1, and the activation of ribosomal protein S6 kinase beta-1 (p70S6K1) which leads to phosphorylation of ribosomal protein S6 (S6) [24]. These processes lead to the translation of mRNA of skeletal muscle proteins [25].

The major protein degradation pathways in skeletal muscle are the ubiquitin-26 S proteosome system (UPS) and the autophagic lysosomal pathway (ALP) [26]. The UPS is an ATP-dependent proteolytic system which removes atrophying muscle cell proteins through poly-ubiquitination of substrates leading to their degradation [27]. Muscle-specific E3 ligases Atrogin-1 and muscle ring finger 1 (MuRF-1) regulate the conjugation of poly-ubiquitin chains during degradation [28].

Dephosphorylation of Akt upregulates transcription factors part of the Forkhead Box O family (FoxOs), which are responsible for the upregulation of Atrogin-1 and MuRF-1 ligases. The activity of FoxOs and E3 ligases have been shown to be upregulated in several models of skeletal muscle atrophy both *in vivo* and *in vitro* [29, 30]. During muscle protein degradation, the catabolic process autophagy is also upregulated, where damaged cell components such as mitochondria, proteins and nuclei are targeted for lysosomal degradation (Figure 3). Vesicles known as autophagosomes engulf cellular particles from skeletal muscle and fuse with lysosomes resulting in

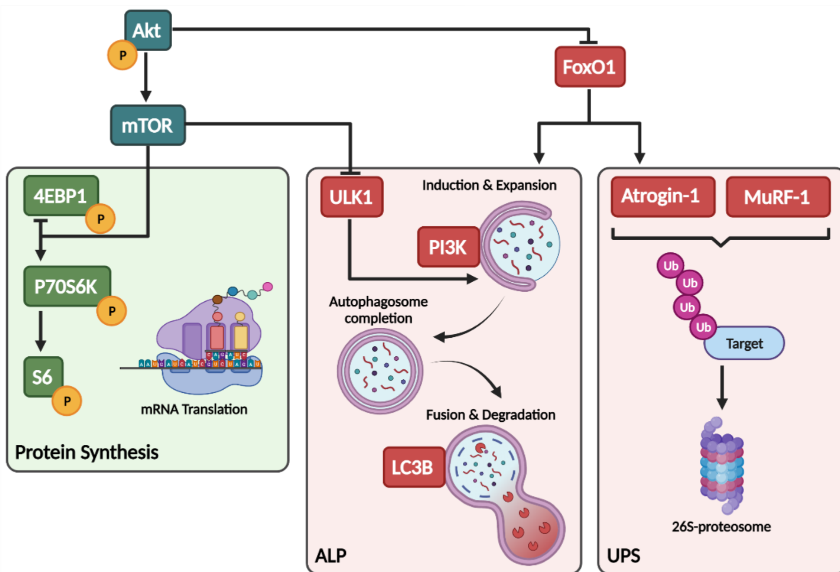

Figure 3. Schematic overview of protein turnover signalling in skeletal muscle. Protein synthesis, autophagosome lysosomal pathway (ALP) and ubiquitin-26 S proteosome-system (UPS). Green show markers of protein synthesis, red show markers of protein degradation markers and blue show upstream regulators. Created with BioRender.

their degradation [31]. The process of autophagy is also regulated by activation of the IGF-1/Akt pathway activation, leading to downstream inhibition of mTOR-mediated inhibitory phosphorylation of unc51-like kinase-1 (ULK1) and FoxO3 [32]. In short, in response to catabolic conditions like fasting, Akt is inactivated leading to the activation of FoxO3, resulting in the induction of transcription of autophagy-related genes such as light chain 3B (LC3B), Bnip3 and ULKs [31]. Reduced IGF-1 levels also lead to AMP-activated protein kinase (AMPK) activation thus inhibiting mTOR, resulting in the active ULK1 complex driving nucleation [33]. Phagophore membranes are associated with Beclin-1 which recruits several complexes including Atgs [34]. This complex produces PI3P, which facilitates the recruitment of acting E3 ligase complex (Atg12, Atg5, Atg10, Atg16L and Atg7), an essential complex for LC3B-II formation via LC3B-I conjugation to phosphatidylethanolamine (PE) [35]. The formation of LC3B-II is crucial in autophagosome maturation [36]. Measurements of LC3B-II/I ratios are used as an indicator or autophagic flux during skeletal muscle atrophy.

Muscle wasting can occur with a lack of anabolic triggers, such as disuse, starvation, aging and reduction in growth factors. Sarcopenia refers to the progressive reduction in muscle mass, strength and function in aging adults [37]. This process contributes to physical impairment and increased risk in falls and disability [38]. The mechanisms underpinning sarcopenia are complex and multifaceted, but can be broadly characterised by a general systemic loss of muscle, with a progressive

reduction in Type II muscle fibers relative to Type I [39]. This in turn contributes to several further risk factors including muscle disuse, reduced protein and caloric intake, reduced growth factor serum concentrations (testosterone, IGF-1) [40, 41]. Furthermore, cachexia is the involuntary loss of skeletal muscle and adipose tissue affecting treatment and mortality in patients with chronic illnesses such as cancer, chronic obstructive pulmonary disease (COPD) and rheumatoid arthritis (RA) [42].

A plethora of studies reveal that chronic inflammatory diseases (CIDs) are associated with increased muscle wasting and systemic elevation of pro-inflammatory cytokines. These inflammatory cytokines are mediators in driving catabolic pathways whilst also downregulating proteolytic pathways [43-45]. This thesis focuses on two chronic inflammatory diseases in particular, RA and COPD, where skeletal muscle wasting are often exemplar complications of disease.

1.2 Chronic Inflammatory Disease

Chronic inflammatory disease is an umbrella term for an array of chronic diseases such as RA, COPD, chronic heart failure (CHF), chronic kidney disease (CKD) and inflammatory bowel disease (IBD). These conditions share common features, including prolonged periods of local and systemic inflammation and elevated pro-inflammatory cytokine and inflammatory mediator profiles. Common symptoms that are shared across CIDs include muscle weakness, chronic fatigue, depression and mood disorders, alterations in weight, susceptibility to recurring infections, myalgia

and pain, significantly reducing patients' quality of life. In addition, age, dietary intake, smoking status, hormone composition and stress are all risk factors associated with increased incidence of CIDs. World Health Organisation (WHO) associate 71% of all deaths globally to chronic disease [46], with CID responsible for 60% of deaths worldwide [47]. Several therapeutic interventions are used in managing and reducing inflammation in CID, such as corticosteroids (cortisone, hydrocortisone, prednisolone) and non-steroidal anti-inflammatory drugs (NSAIDs) (aspirin, ibuprofen, naproxen) [48].

Although some characteristics of acute inflammation persist as these chronic diseases develop, there are features of chronic inflammation that are entirely distinct. Usually, acute inflammation has a short-term duration and typically, initially involves the innate immune system which acts as a barrier and first line defence against infectious agents and pathogens. The immediate, adaptive response of the innate immune system involves a cascade of processes with mediators such as neutrophils and macrophages playing a crucial role [49]. Following infection, resident macrophages activate and surface receptors known as pattern recognition factors (PRFs) identify pathogen-associated molecular patterns (PAMPs) on pathogens, and release pro-inflammatory cytokines and chemokines [50]. These chemical factors recruit neutrophils, basophils and leukocytes to the site of infection where they exert their immune effects through processes such as phagocytosis and lysozyme degradation [50]. These innate acute inflammatory responses pave the

way for a more established adaptive immunity, which in some cases results in chronic inflammatory responses. Chronic inflammation is frequently characterised a persistent recruitment of macrophages, mononuclear leukocytes and lymphocytes with progressive tissue damage [51]. In this chronic setting, inflammatory chemokines and cytokines (such as IL-1, IL-6 and TNF-α) are sustained where they also promote local fibrosis at sites of inflammation as a result of prolonged fibroblast recruitment and hyper-proliferation [52]. This prolonged inflammatory response affects multiple systems including the endocrine, nervous, and reproductive system [53, 54]. A prevalent consequence of chronic inflammation is skeletal muscle wasting and muscle weakness [55]. Complications of muscle wasting in CID include increased risk of falls and fractures, disability and reduced mobility, poorer quality of life, increased risk of hospitalisation and therefore higher risk of secondary hospital acquired illness, and increased risk of developing other chronic disease such as heart disease [1]. In turn, there is significant socioeconomic burden for patients with CID resulting from direct healthcare costs (e.g., therapeutics and care) and indirect non-healthcare costs (e.g., disability payments and work productivity), which increase significantly with disease progression [56-58].

Mechanisms underpinning the pathophysiology of skeletal muscle wasting in CID remain poorly understood. Consequently, a greater understanding of this process is required to design effective therapeutic intervention aimed at preventing inflammatory muscle loss.

1.2.1 *Rheumatoid Arthritis*

Rheumatoid arthritis, affecting 1-2% of the global population, is a chronic systemic autoimmune disorder, characterised by progressive joint destruction, with synovitis, loss of cartilage and bone that result in pain, joint deformity and disability [59]. Development of RA is associated with several risk factors such as environmental (viral and bacterial infections, smoking) and genetic (hormonal and epigenetic), [60] and disproportionally effecting the elderly and women [61]. Diagnosis of RA is achieved through combined measures of joint assessment using MRI and ultrasound, elevated markers of inflammation (C-Reactive Protein, Erythrocyte Sedimentation Rate, Rheumatoid Factor and cyclic citrullinated peptide) and patient symptoms and medical history [62].

Synovitis, the destruction of the synovial joint, is a result of inflammatory and immune cell infiltration into the synovium, which is reflected in swelling of the joints in RA patients. The synovium is formed by two types of synoviocytes, A (macrophage-like) or B (fibroblast like, FLS), with a synovial membrane that allows diffusion of nutrients to the joint [59]. Anti-citrullinated protein antibodies (ACPA) are a collection of antibodies (IgG, IgA, IgM) that recognise citrulline in proteins, and is associated with increased disease severity in RA [59]. In RA, monocytes and macrophages of the innate immune system infiltrate the synovial membrane and are central in driving disease pathophysiology through the production of potent inflammatory mediators such as TNF-α and IL-1β [63]. Monocytes and macrophages

with citrullinated Grp78 receptors are bound by ACPA which leads to enhanced TNF-α production and NF-κB activity [64]. Patients with APCA-associated RA are also more prone to M1 and M2 polarisation imbalances, driving osteoclast production ultimately leading to bone erosion [65]. Furthermore, infiltration of immune mediators such as leukocytes and T and B-lymphocytes into the synovial joint interact with fibroblast-like synoviocytes (FLSs) drive local joint destruction and persistence of inflammation [59]. Cartilage destruction is driven by factors secreted by these FLSs', such as matrix metalloproteinases (MMPs) and collagenase. Infiltration of these inflammatory mediators drives a cascade of pro-inflammatory cytokine and chemokine secretion, establishing a feedback loop paving the way for more leukocyte infiltration and chronic inflammation [59].

1.2.2 Peripheral complications of RA

Muscle wasting is a significant component of RA contributing to a, increased sedentary lifestyle, reduced physical activity and a poorer quality of life. Muscle wasting in RA patients may occur due to various reasons, such as malnutrition, disuse, sarcopenia and, inflammatory-driven and glucocorticoid-driven muscle atrophy. Pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IFN-γ are found in both the synovial joint and the plasma of RA patients, promoting both local and systemic inflammation. As a result of systemic inflammation, 40% of RA patients experience extra-articular consequences such as osteopenia, cardiac disease,

pulmonary complications, and myopathy, which are predictors of increased mortality [66, 67]. Increased levels of inflammatory cytokines in RA patients have been implicated in driving muscle wasting through several mechanisms. Glucocorticoids (GCs) such as prednisone are often used in the treatment of RA due to their potent anti-inflammatory properties, however their long-term use is also associated with undesirable side effects, including muscle atrophy.

1.2.3 COPD

Chronic obstructive pulmonary disease is a progressive lung disorder characterised by chronic inflammation and irreversible remodelling of the airways leading to airflow limitation. The third leading cause of death worldwide by the WHO [68], COPD is a major burden on public health and significantly affects patients' quality of life. Several risk factors are associated with the development of COPD and whilst smoking is the primary cause, others include environmental exposure to chemicals, fumes and lung irritants, infectious agents and low socioeconomic status [69]. Underpinning the restricted airflow associated with COPD are two pathological processes including narrowing of the airways as a consequence of bronchitis, and destruction of the parenchyma of the lungs due to emphysema development. During emphysema development, macrophages and neutrophils are recruited into the small airways, releasing proteases such as elastase, which break down elastin fibers, leading to a reduced elasticity of the airways and loss of alveolar cells in the lungs

leading to increased airspace [70]. The role of proteases in development of emphysema rests on clinical and experimental observations. Moreover, previous mouse models have shown intra-trachaelly instilled elastase is sufficient to induce emphysema [71]. Reactive oxygen species (ROS) are released by alveolar macrophages in the airways in response to triggers such as cigarette smoke, which contribute to elevated NF-κB activation, subsequent inflammation in both the lungs and systemically, increased DNA damage and cell death [72]. The loss of the alveoli membranes results in a reduced gas exchange, often followed by hypoxemia which leads to shortness of breath, coughing and increased heart rates [73].

Exacerbations of COPD, which are defined as episodes of intensified disease symptoms, are associated with decreased survival and increased disease progression [74, 75]. Classification of COPD patients using the GOLD (Global Initiative for Chronic Obstructive Lung Disease) system uses exacerbation history, along with symptoms and airflow obstruction to determine risk status. There are 4 classifications of GOLD based on FEV_1 (Forced Expiratory Volume) output; GOLD A (low risk/low symptoms, > 80% FEV_1), GOLD B (low risk/high symptoms, 50-79% FEV_1), GOLD C (high risk/low symptoms, 30-49% FEV_1), and GOLD D (high risk/high symptoms, < 30% FEV_1), which are used to establish patient risk of frequent exacerbations and disease progression [76]. Exacerbations of COPD, commonly induced by bacterial of viral infections [77], are usually accompanied by pulmonary and systemic inflammation, which often lead to the development or acceleration of extra-pulmonary consequences, including

skeletal muscle wasting [78, 79]. This aspect can be modelled by instilment of bacterial components such as lipopolysaccharide (LPS) into the lungs of emphysematous mice, which has previously shown to mimic aspects of acute exacerbations in COPD [80].

Extra-pulmonary consequences of COPD such as muscle wasting are associated with increased risk of hospitalisation, mortality and poor quality of life [81]. Depending on disease stage, skeletal muscle wasting is observed in 20-40% of patients with COPD [82, 83], which ultimately contributes to reduced exercise capacity and health status, is seen largely in patients with emphysema [84, 85] and those with frequent exacerbations [86]. Although the mechanisms underpinning COPD-associated muscle wasting remains unclear, several potential triggers of muscle wasting have been identified, which include disuse, hypoxemia, malnutrition, inflammation and glucocorticoids (GCs) [73]. Glucocorticoids are often used for their anti-inflammatory properties in the treatment of COPD, in particular during an acute exacerbation of disease state. However, GCs influence muscle metabolism themselves and therefore add further complexity to muscle wasting in COPD.

1.3 Glucocorticoids

1.3.1 Introduction

Glucocorticoids (GCs) are lipophilic, pleotropic steroid hormones that play an essential role in energy metabolism [87]. Glucocorticoids are involved in a

multitude of pathological processes including energy metabolism (glucose, protein, and fat), growth, cognitive functions, reproduction and development, water and electrolyte balance and cardiovascular function, as well as having potent anti-inflammatory and immune-suppressive actions [87-93]. Secreted by the adrenal glands, endogenous GCs (such as cortisol and corticosterone) are regulated by the hypothalamus-pituitary-adrenal (HPA) axis and are released in a circadian rhythm. The release of GCs by the HPA axis is also heightened in response to psychological and physiological stress, such as injury and infection, and elevated levels of pro-inflammatory cytokines such as TNF-a, IL-1, IL-6 and IL-8 [94, 95]. In addition to endogenous regulation, therapeutic glucocorticoids (dexamethasone, prednisolone, hydrocortisone) are used widely in the treatment of chronic inflammatory disease. Despite their potent anti-inflammatory actions, application of GCs are associated with a variety of undesirable side effects, such as muscle wasting, osteoporosis and the GC excess disorder Cushing's syndrome [96, 97].

1.3.2 Therapeutic Steroids and GC excess

In 1950, Hench, Kendall and Riechstein received the Nobel Prize for their discovery and isolation of glucocorticoids from bovine adrenal glands [98]. These were labelled A-F. Shortly after, it was shown that an RA patient administered E (cortisone) resulted in spontaneous remission of disease [98]. Exogenous or synthetic GCs are extensively used in the treatment of autoimmune and

inflammatory disorders due to their low cost and anti-inflammatory properties. As a result, chemists developed an assortment of new synthetic GCs possessing longer-half lives than endogenous GCs. Alterations in the structures of synthetic GCs altered the potency of them greatly, such as dexamethasone potency being 25 times higher than that of prednisolone [99]. Typically, the route of administration of GCs depends on the type of disorder being treated and disease severity or activity. For instance, patients with COPD are more likely to receive inhaled GC therapy with the intention of reducing localised inflammation in the lungs, whereas RA patients may have oral prednisolone, or where pain is highly localised, for instance an injection of prednisolone into the joint. Patients suffering from AE-COPD or in a worsened disease state of RA may also be prescribed oral daily GCs to reduce systemic inflammation. Despite GC efficacy, long term application of therapeutic GCs are associated with an array of undesirable side effects, such as muscle wasting, osteoporosis, or GC excess known as Cushing's syndrome [100].

Harvey Cushing first describes Cushing's syndrome in 1912, as glucocorticoid excess leading to a range of clinical manifestations such as increased adiposity in the trunk, shoulders and face, wasting in the limbs (both skeletal muscle and adipose tissue), hypertension, lethargy, depression, osteoporosis and infertility [101, 102]. Associated with high mortality [103], Cushing's syndrome occurs as a result of a loss of circadian rhythm of GC secretion from adrenal glands in addition to dysregulation of the negative feedback loop controlled by the HPA axis. The most

common cause of the dysregulation is exogenous treatment of GCs, however there are other causes of Cushing's as a result of HPA dysregulation endogenously. In brief, Cushing's syndrome can be divided into one of two categories: ACTH-independent and ACTH-dependent Cushing's. Cushing's disease arises from ACTH-dependent Cushing's syndrome, usually as a result of a pituitary adenoma, or ectopic ACTH producing tumours [102]. Adrenal tumours are associated with ACTH-independent Cushing's, however the most common cause of Cushing's syndrome is treatment with therapeutic GCs [104].

1.3.3 Structure, synthesis, and function of GCs

Steroid hormones including GCs are derived from cholesterol metabolism and share a basic structure of 17-carbon androstane structure with three cyclohexane rings and a cyclopentane ring [105]. Glucocorticoids in particular have a hydroxyl group at carbon-11, and their potency and affinity are dependent on the positioning of different methyl, ester, hydroxyl and ketone groups on different carbons [106]. Secreted by the adrenal glands, GCs are regulated by the HPA axis. Under normal conditions, the regulation of GCs release is controlled by the HPA axis in a circadian manner, with higher levels generally seen during the morning in humans and the evening in nocturnal animals [107]. The hypothalamus synthesises and releases corticotropin-releasing hormone (CRH) which upon binding to the CRH type I receptor in the anterior pituitary glands, induces adrenocorticotropic hormone

(ACTH) release into circulation. Following binding of ACTH to the ACTH receptor in the adrenal cortex, GCs are synthesised and released [108]. Using a negative feedback loop, the HPA axis then downregulates the secretion of CRH and ACTH to reduce GC secretion once circulating levels are high (Figure 4). In response to stress triggers, such as starvation, trauma or inflammation, ACTH levels increase independently of circadian rhythmicity, which drives cortisol synthesis and secretion [109]. Inflammatory cytokines have been implicated in the activation of the HPA axis, ultimately leading to increased levels of GCs released into the circulation to prevent cytokine storms. Further detail of the impact of cytokines on the HPA axis and GC release can be found in Chapter 2 (page 77).

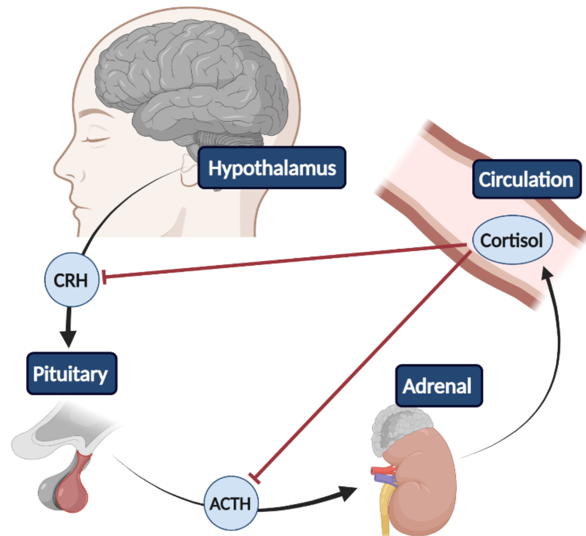

Figure 4. Schematic of the hypothalamus-pituitary-adrenal (HPA) axis. The hypothalamus releases CRH, which increases ACTH secretion from the pituitary gland. The adrenal glands synthesise and release cortisol into circulation in response to ACTH. Red arrows represent inhibition of secretion by cortisol with a negative feedback loop of the HPA axis. Created with BioRender.

Glucocorticoids regulate carbohydrate homeostasis through several mechanisms, including promotion of hepatic gluconeogenesis and increased liver glycogen storage, reduction of glucose uptake and glycogen storage in skeletal muscle and inhibition of insulin secretion from the pancreas [110, 111]. These changes in carbohydrate metabolism are of particular importance during times of stress, such as fasting and starvation, in order to provide the brain with sufficient glucose to maintain brain function [112]. Moreover, GCs have profound effects on lipid metabolism, such as increasing adipocyte differentiation and lipolysis which leads to increased circulating fatty acids [113]. An additional contributor to GCs ability to increase circulating fatty acids which ultimately lead to central obesity, GCs increase very low density lipoproteins (vLDLs) following elevated triglyceride production and secretion in the liver via enhanced expression of fatty acid synthase [114]. Glucocorticoids have a profound effect on protein metabolism, most importantly in skeletal muscle, through eliciting their anti-anabolic and catabolic effects (detailed below, 1.2.3.). After a stress response, such as starvation or disease, circulating GCs are released and exert their catabolic effects through inhibiting muscle protein synthesis and driving proteolysis, resulting in the subsequent release of amino acids, of which are required to provide substrate for hepatic gluconeogenesis [115]. Sustained exposure to elevated GCs, either endogenously or therapeutically, is associated with skeletal muscle wasting and a reduction in strength. A comprehensive description of the mechanisms underpinning GCs actions

on skeletal muscle can be found in Chapter 2 (page 77) and Chapter 4 (page 173). In summary, binding of GCs to the GR reduces downstream activation of the IGF-1 signalling pathway, including Akt/mTOR activation [116], inhibiting protein synthesis mediated by 4E-BP1 and S6 kinase [116]. Furthermore, GCs increase anti-anabolic signalling mediated by the induction of myostatin, DNA damage-inducible transcript 4 protein (REDD1) and kruppel-like factor 15 (KLF15) [117, 118]. Proteolysis is upregulated in response to GR activation via increased FoxO1 and FoxO3 gene expression, leading to elevated transcription of muscle-specific E3 ligases Atrogin-1 and MuRF1 [29, 119].

1.3.4 Regulation of GC actions

Following GCs release into the circulation, they bind to the corticosteroid-binding globulin (CBG) α_2 with high affinity, rendering GCs inactive [120]. Around 90% of GCs found in circulation are inactive and bound to CBG and albumin, with the remaining 10% unbound [121]. Lipophilic unbound GCs freely diffuse across cell membranes into the cytosol of the cell where they bind to the glucocorticoid-receptor to exert their function. Further detail on GR binding and GC action can be found in Chapter 4 (page 156).

In brief, the GR is encoded by the nuclear receptor subfamily 3 group c member 1 (NR3C1), which can have numerous promoter regions, resulting in varying GR protein isoforms and therefore multiple ligand binding sites allowing GCs, other

steroids, or selective glucocorticoid receptor modulators (SEGRMs) to bind to the GR [122]. Located in the cytosol, unbound monomeric GR forms a heterocomplex with heat shock protein-90 (hsp90), heat shock protein-70 (hsp70), p23 and immunophilin [123]. Upon GC binding, the hsp complex dissociates following GR conformational change, and is replaced with an alternative complex including p23 and FKPB51 [124] . This new complex allows for efficient nuclear translocation of the GR, in addition to the binding of nucleoporin, through the nuclear pore complex [125]. Once inside the nucleus, the activated GR is either transported back into the cytoplasm, modulate other transcription factors via tethering, or act as a transcription factor itself to trans-repress or trans-activate gene transcription [126] (Figure 5). Classic signalling is characterised by ligand bound or unbound GR binding to glucocorticoid binding sites (GBS) which can contain glucocorticoid responsive elements (GRE) [126]. Upon the GR binding to these GREs as homodimers, co-activators or co-repressors are then recruited, leading to reduced transcription of either pro-inflammatory genes such as NF-κB or increased transcription of anti-inflammatory genes such as *TSC22D3* (coding for glucocorticoid-induced leucine zipper, GILZ) [127-129]. Further detail into the genes regulated by GR signalling can be found in Chapter 4 (page 156). Trans-repression may also result from a GR monomer tethering to inflammatory transcription factors (such as NF-κB, AP1 and STAT) and the subsequent recruitment of co-repressors trans-repress pro-inflammatory genes [127] (Figure 5). This is a

simplified overview of the genomic effects of GR activation and has been previously reviewed extensively [127].

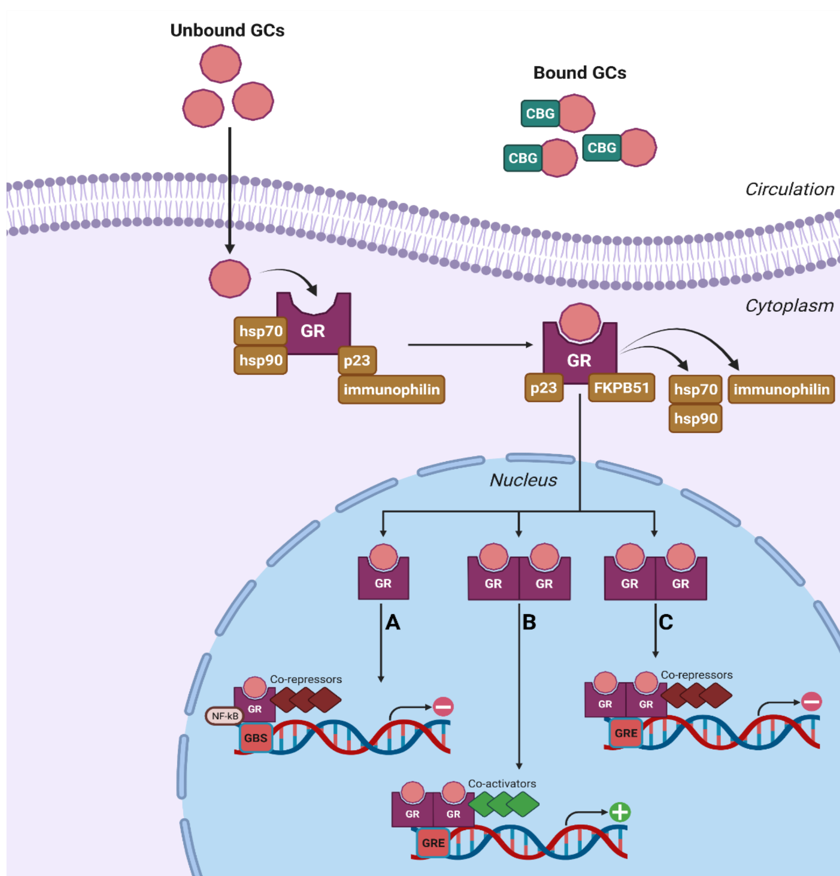

Figure 5. Overview of GR-activation and function. Unbound circulating GCs move across the phospholipid bilayer into the cytoplasm and bind to the GR. Following dissociation of hsp complex, activated GR translocates to the nucleus where they induce their genomic effects. (**A**) Monomeric GR tethered to inflammatory transcription factors, recruits co-repressors, binds to GBS and reduces pro-inflammatory gene transcription. (**B**) GR homodimers recruit co-activators and bind to GRE to induce anti-inflammatory gene transcription. (**C**) GR homodimers recruit co-repressors, bind to GRE to inhibit pro-inflammatory gene transcription. Created with BioRender.

1.3.5 Pre-receptor Metabolism of glucocorticoids

Peripheral exposure to GCs is mediated through pre-receptor metabolism by the 11 beta-hydroxysteroid dehydrogenase (11β-HSD) enzymes. 11β-HSD1 is predominantly found in abundance in tissues such as adipose tissue, liver, brain and skeletal muscle [130]. This bi-directional enzyme predominantly converts inactive glucocorticoids (cortisone and 11-dehydrocorticosterone) to their active counterparts (cortisol and corticosterone) through an oxoreductase manner using the high concentrations of NADPH found in the endoplasmic reticulum (ER) membrane [2] (Figure 6). The conversion of inactive GCs to their active counterparts results in an increased local active GC level within tissue [131], however, 11β-HSD1 expression has also been shown to be increased in response to inflammation, further increasing the levels of active GCs [130]. In contrast, 11β-HSD2 is found primarily in tissues expressing the mineralocorticoid receptor (MR), such as the kidney [132], where it only converts active GCs to their inactive counterparts [133] (Figure 6). Following inactivation via 11β-HSD2, inactive GCs are released into circulation, allowing renal clearance of active GCs, thus protecting the kidneys from GC-induced MR activation which could lead to hypertension [133, 134]. Further detail of the role of pre-receptor metabolism in skeletal muscle atrophy is found in Chapter 2 (page 80) and Chapter 4 (page 179).

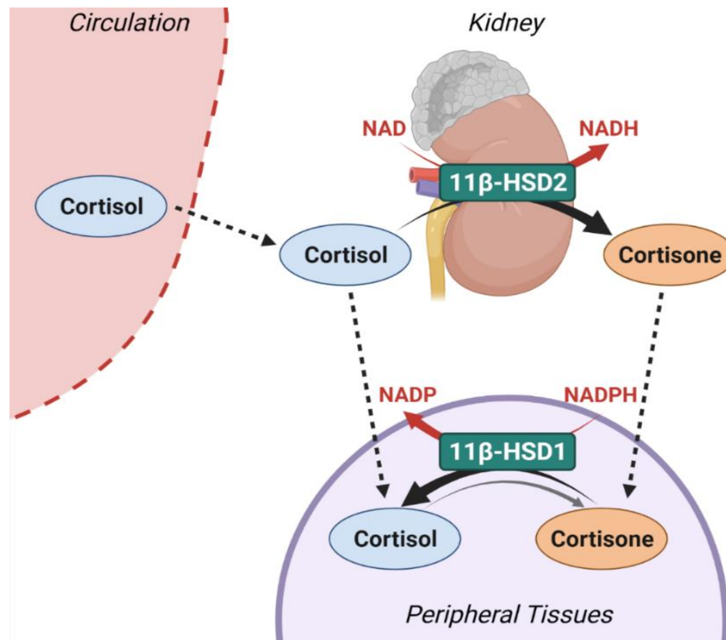

Figure 6. Pre-receptor regulation of GCs by 11β-HSD enzymes. Circulating active GCs are deactivated in the kidneys (and other organs expressing MR) by 11β-HSD2. Inactive GCs are activated by 11β-HSD1 in peripheral tissues to their active counterparts. Created with BioRender.

Previous reports of a patient suffering from Cushing's disease showed protection from the typical Cushing phenotype due to a functional defect in 11β-HSD1 activity, further highlighting the importance of pre-receptor metabolism in disorders associated with GC-excess [135, 136]. Pharmacological inhibitor of 11β-HSD1 carbenoxolone has since been explored profusely and has shown improved insulin sensitivity and inhibition of GC-induced lipolysis [131, 137]. Following, potent selective 11β-HSD1 inhibitors, such as INCB013739 and MK0916, have been developed and have shown promising effects such as reduced hepatic

gluconeogenesis in patients with type 2 diabetes, and a reduction in blood pressure in hypertensive patients [131, 138]. Morgan *et al*., has previously highlighted the importance of 11β-HSD1 in driving muscle wasting during GC excess, with mice with a genetic ablation of 11β-HSD1, treated with corticosterone, showed a protective phenotype against muscle atrophy [136]. Their data relayed that GC excess reduced muscle mass, strength, and increased expression of muscle atrophy markers such as MurF-1, Atrogin-1, Mstn and FoxO1, however in combination with a global deletion of 11β-HSD1, these effects were abrogated [136]. In summary, therapeutic inhibition of 11β-HSD1 in chronic inflammatory disease may offer beneficial protection against GC-induced muscle atrophy, by reducing local amounts of active GCs in the skeletal muscle.

1.4 Objectives and outline of this thesis

It is currently unclear what the role of 11β-HSD1 is in GC-mediated muscle atrophy in inflammatory disease. Therefore, the overall objective in this thesis is to investigate the role of 11β-HSD1 in glucocorticoid-driven skeletal muscle atrophy in chronic inflammatory disease using models of COPD and RA. Chapter 2 of this thesis describes in detail the clinical and experimental evidence implying a role of inflammation in muscle wasting, including cellular processes and causal mechanisms. Systemic and tissue-specific inflammation is discussed, and the direct and indirect impacts of inflammation on muscle in cachexia are detailed. Using a compound

model of LPS-induced acute exacerbation of emphysematous mice (COPD) with a global deletion of 11β-HSD1, Chapter 3 explores the contribution of 11β-HSD1 in GC-associated skeletal muscle wasting. Non-invasive µCT scans were used to confirm emphysema development and determine skeletal muscle volume changes in a longitudinal approach. Combined with *ex vivo* and *in vitro* experiments, the impact of systemic deletion of 11β-HSD1 on muscle protein and myonuclear turnover is explored during experimental COPD acute exacerbation. In Chapter 4 therapeutic GCs and their effects on muscle and bone are outlined, with detail highlighting their anti-inflammatory properties, and the interaction between inflammation and GCs in both muscle and bone. Chapter 5 investigates the effects of therapeutic corticosteroid treatment in wild-type (WT) mice and mice with experimental polyarthritis (genomic overexpression of human TNF-α) to determine the effects of GCs on muscle and bone. In order to determine what role 11β-HSD1 has in this model, Chapter 6 uses the TNF-tg mouse model combined with a global deletion of 11β-HSD1, treated with oral glucocorticoids once more, with particular focus on skeletal muscle wasting. Finally, in Chapter 7 the implications of the experimental findings are discussed, with specific attention to similarities and differences between COPD and RA), with focus on inflammation, glucocorticoids and 11β-HSD1 involvement. Gaps and limitations in current research and approaches are discussed in addition to possible therapeutic strategies to reduce or prevent muscle wasting in GC-treated chronic inflammatory disease.

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Chapter 2 - Inflammation and Skeletal Muscle Wasting During Cachexia

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Abstract

Cachexia is the involuntary loss of muscle and adipose tissue that strongly affects mortality and treatment efficacy in patients with cancer or chronic inflammatory disease. Currently, no specific treatments or interventions are available for patients developing this disorder. Given the well-documented involvement of pro-inflammatory cytokines in muscle and fat metabolism in physiological responses and in the pathophysiology of chronic inflammatory disease and cancer, considerable interest has revolved around their role in mediating cachexia. This has been supported by association studies that report increased levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in some, but not all, cancers and in chronic inflammatory diseases such as chronic obstructive pulmonary disease (COPD) and rheumatoid arthritis (RA). In addition, preclinical studies including animal disease models have provided a substantial body of evidence implicating a causal contribution of systemic inflammation to cachexia. The presence of inflammatory cytokines can affect skeletal muscle through several direct mechanisms, relying on activation of the corresponding receptor expressed by muscle, and resulting in inhibition of muscle protein synthesis (MPS), elevation of catabolic activity through the ubiquitin-proteasomal system (UPS) and autophagy, and impairment of myogenesis. Additionally, systemic inflammatory mediators indirectly contribute to muscle wasting through dysregulation of tissue and organ systems, including GCs via the hypothalamus-pituitary-adrenal (HPA) axis, the

digestive system leading to anorexia-cachexia, and alterations in liver and adipocyte behaviour, which subsequently impact on muscle. Finally, myokines secreted by skeletal muscle itself in response to inflammation have been implicated as autocrine and endocrine mediators of cachexia, as well as potential modulators of this debilitating condition. While inflammation has been shown to play a pivotal role in cachexia development, further understanding how these cytokines contribute to disease progression is required to reveal biomarkers or diagnostic tools to help identify at risk patients, or enable the design of targeted therapies to prevent or delay the progression of cachexia.

Keywords: cachexia, inflammation, muscle wasting, atrophy, cancer, COPD, cytokines

2.1 Cachexia In Cancer and Chronic Disease

Cachexia is a complex metabolic syndrome resulting in severe weight loss, anorexia, and asthenia that occurs in many types of cancer and chronic inflammatory diseases [1]. The nature of this weight loss is characterized by a pronounced systemic muscle wasting and weakness and includes marked loss of fat mass. Consequently, cachexia remains a significant co-morbidity in many diseases increasing mortality, reducing quality of life, and representing a major burden on healthcare providers. However, despite its impact in human disease the mechanisms that underpin cachexia remain poorly defined. In this review, we provide an overview and explore the latest insights into the contribution of systemic inflammation in cachexia, including both direct and indirect mechanisms of inflammatory muscle wasting.

Cachexia is highly prevalent in various types of cancers, with estimates of 30% in lung and head and neck cancer, and 41–45% in pancreatic and liver cancer, respectively [2]. In addition, a high prevalence of cachexia has also been described for patients with chronic illnesses, including chronic heart failure (CHF; 10–39%) [3, 4], chronic kidney disease (CKD; 30–60%) [5], chronic obstructive pulmonary disease (COPD; 5–33%) [6-8], and rheumatoid arthritis (RA; 19–32%) [9] (although RA is often debated as being a cachectic disease as weight loss is not always a defining feature; [10]. Cachexia is associated with decreased survival and quality of life in these conditions [11-13], and poor clinical outcome as is illustrated by increased

postoperative mortality for cancer [14] and CHF [15] and decreased response to radiation-, chemo-, and immunotherapy in the presence of cachexia [16].

Diagnostic criteria for cachexia include weight loss in the presence of underlying illness of >5% in ≤12 months, or weight loss >2% in individuals with a low body-mass index (<20 kg/m^2) or low muscle mass [17], and the presence of decreased muscle strength, fatigue or anorexia, and abnormal biochemistry [1]. Interestingly, the latter refers to the presence of increased inflammatory serum markers like interleukin-6 (IL-6) and C-reactive protein (CRP), suggesting inflammation as a shared characteristic of cachexia in these distinct conditions. Indeed, several clinical studies have reported elevated levels of inflammatory markers in cancer patients with cachexia. In non-small cell lung carcinoma, CRP, IL-6, and interleukin 8 (IL-8) serum levels were increased in cachectic compared to non-cachectic lung cancer patients [18]. Riccardi *et al*. (2020) recently highlighted changes in plasma concentrations of markers of systemic inflammation in patients with cancer cachexia compared to weight-stable cancer patients. A positive correlation between pro-inflammatory cytokines and fatty acid lipid profile was reported in patients with gastrointestinal cancer cachexia, accompanied with augmented levels of CRP, and elevated levels of pro-inflammatory cytokines IL-6, tumor necrosis factor-alpha (TNF-α), and IL-8 [19]. In patients with gastro-esophageal cancer, multiple regression analysis identified dietary intake and serum CRP concentrations as independent variables in determining the degree of weight loss, with a higher predicted effect than disease

stage [20]. Based on these and other studies, it is now recognized that a systemic inflammatory response is associated with weight and muscle loss and poorer outcomes in patients with cancer [21], and can be applied in identifying the various stages of cachexia [22]. A recent systematic review by Abbass *et al*. (2019) revealed a consistent correlation between systemic inflammation and low skeletal muscle index determined through CT and DEXA scans in patients with various types of cancer, further implicating a link between inflammation and skeletal muscle mass loss in cancer cachexia [23].

Similarly, a correlation between cachexia and inflammation has also been described in chronic diseases. Cachectic patients with CHF often exhibit elevated levels of pro-inflammatory cytokines such as TNF-α, IL-6, interleukin-1 (IL-1), and interferon-γ (IFN-γ), in addition to glucocorticoids (GCs) [24]. These molecules act as signaling ligands to directly and indirectly impact muscle and adipose tissue metabolism in favor of wasting [25]. Patients with cardiac cachexia also exhibit elevated levels of Angiotensin II (Ang II), which has been shown to increase the levels of these pro-inflammatory cytokines [26, 27], and induce cachexia [26]. In CKD patients, increased expression of TNF-α and IL-6 is detected in skeletal muscle [28], and cachexia is accompanied by increases in circulating TNF-α and IL-6 in addition to CRP [29], which often occur in the presence of malnutrition, and therefore is referred to as malnutrition-inflammation-cachexia syndrome [30]. In patients with CKD, cachexia was not only associated with elevated levels of CRP, but also increased

fibrinogen, and reduced cross-sectional area (CSA) of muscle fibers and fat mass [31]. Cachexia development in COPD has also been associated with the presence of systemic inflammation. COPD patients with unintentional weight loss and skeletal muscle mass loss showed elevated serum cytokine levels including TNF-α and IL-6 compared to stable-weight patients [32, 33]. Disease-specific characteristics of COPD have been implicated as the driver of inflammation, such as increased TNF-α levels as a result of hypoxemia resulting from reduced lung function [34], or increased systemic inflammation secondary to disease exacerbations in COPD [35]. Respiratory infections and subsequent pulmonary inflammation are a frequent cause of exacerbation, and these episodes are accompanied by catabolic changes in skeletal muscle [36], and considered as a potentially accelerating phase in COPD cachexia [37]. It is well established that patients with RA have elevated levels of inflammatory cytokines, specifically TNF-α, IL-1β, and IL-6, and are found directly at the synovial joint or found to be released into the plasma, stimulating both local and systemic inflammation [38-40]. The latter have been implicated in rheumatoid cachexia, with reports of reduced lean body mass (LBM) correlating with elevated serum levels of IL-6 and CRP [41, 42]. In other chronic inflammatory conditions than RA, like Crohn's disease, correlations with weight loss have also been described [43-48], indicating that the relation between systemic inflammation and cachexia may extend beyond the diseases described here. Importantly, a correlation between circulating inflammatory cytokines and cachexia may not be detected in all patients, as a result

of the rapid systemic clearance, or the presence of additional triggers of muscle and weight loss in these complex conditions, such as malnutrition in CDK [5] and COPD [49], or hypoxemia in COPD [50]. In addition, exacerbations in conditions like RA, COPD, and Crohn's disease activity may be associated with transient increases in systemic inflammation, followed by sustained loss of body and muscle mass, which are not recovered as a result of other disease-related impairments in, e.g., inactivity, hypoxemia, or malnutrition [50].

The observed correlation between inflammation with cachexia across the various diseases described above is the basis for including serum markers of increased inflammation as a criterion in the definition of cachexia. Importantly, beyond its use as a clinical hallmark, inflammation has been investigated as a potential driver of cachexia, fueling a number of studies investigating the causal involvement of inflammation and underlying mechanisms by which it contributes to cachexia. An overview of these reports is presented below. To facilitate their discussion, a description of the processes that govern muscle mass is provided first.

2.1.1 <u>Cellular Processes That Determine Muscle Mass</u>

Maintenance and modulation of skeletal muscle mass has been attributed to two processes: protein turnover and myonuclear turnover. Under normal conditions, these processes are maintained in homeostasis, however during skeletal muscle wasting the balance within these processes shift in favor of muscle wasting,

through the inhibition of muscle protein synthesis (MPS), activation of muscle protein degradation, reduction in myonuclear accretion, or increased myonuclear loss. Protein turnover is a dynamic process determined by protein synthesis and degradation and is mediated through transcriptional, translational, and post-translational mechanisms [51]. One important regulatory circuit of protein turnover is the insulin growth factor-1(IGF-1)–phosphoinositide−3−kinase (PI3K)–Akt/protein kinase B (PKB)–mammalian target of rapamycin (mTOR) pathway. Akt is the key mediator stimulating protein synthesis through mTOR activation, while inhibiting protein degradation through phosphorylation of transcription factor (TF) Forkhead box O (FoxO), leading to its cytoplasmic retention where it is inactive [52]. Translational capacity in skeletal muscle is regulated through eukaryotic initiation factors (eIFs) and ribosomal S6 kinase (P70S6K). Formation of the eIF4F complex is a rate-limiting step in initiation of the mRNA translation process [53], while phosphorylation of P70S6K facilitates ribosomal biogenesis and translation [54].

Skeletal muscle is the largest latent reservoir of amino acids, which are mobilized by increased proteolysis of mainly muscle contractile proteins to provide energy or precursors for protein synthesis to other vital organs [55]. Muscle protein degradation increases as a physiological response to starvation but is also activated during pathological catabolic states that accompany inflammation and cachexia. Control of muscle mass is highly regulated by proteolytic enzyme systems including the ubiquitin-proteasomal system (UPS), autophagy-lysosomal pathway (ALP),

caspases, and calpains. The UPS is involved in the removal of specific proteins for degradation following marking with ubiquitin through specific a sequential process catalyzed by ubiquitin-activating enzymes (E1), conjugating enzymes (E2), and ligating enzymes (E3) [56]. It is an enzymatic process initiated by E1 enzymes activating the ubiquitin which is transferred to the E2 ubiquitin-conjugating enzyme [57]. The E2 ubiquitin complex binds to E3 protein ligases that recognize substrate proteins that will be ubiquitinated. Polyubiquitinated proteins are transferred in an ATP-dependent manner to 26S proteasome complexes in which they are degraded. E3 Ub ligases are postulated as rate-limiting factors in this pathway [58], and a number of muscle enriched E3 Ub-ligases have been described, including Atrogin-1 and MuRF1. The ALP is essential for removal of misfolded or aggregated proteins and damaged parts of the cell to prevent accumulation of toxic or abnormal organelles and proteins. In addition, it enables the breakdown of proteins to produce amino acids by skeletal muscle that can be utilized in other tissues during catabolic periods, such as starvation [59]. There are three types of autophagy described in mammals; macroautophagy, microautophagy, and chaperone-mediated autophagy. Although all three processes are distinct from one another, these mechanisms ultimately lead to lysosomal degradation of cargo and recycling of breakdown products [60]. Calpains and caspases are families of cysteine proteases, and their proteolytic activity is increased during cellular necrosis or apoptosis. They have been implicated in suspending cell function through disabling signal transduction molecules by

enzymatic cleavage at specific amino acid residues in a host of cells [61], but their overall relevance to increased muscle proteolysis in muscle atrophy is not yet clear. As will be highlighted in subsequent sections, both decreases in MPS and increases in proteolysis have been implicated in the loss of muscle mass in cachexia.

Myonuclear turnover is another important process involved in muscle homeostasis and is the balance between myonuclear accretion and apoptosis. Myonuclear accretion is the final step in post-natal myogenesis [52], and relies on satellite cells (SCs), the local precursor cells of skeletal muscle [62]. During muscle regeneration, SCs are activated and proliferate, which occurs through asymmetric cell division, resulting in two distinct myoblast populations. A portion of these myoblasts returns into quiescence to prevent depletion of precursor cells. The other population of myoblasts terminally differentiate and fuse with muscle fibers (or myotubes *in vitro*). Committed SCs highly express PAX7 and MYF5, which decreases during differentiation. MyoD is an important myogenic regulator during proliferation and early differentiation. Conversely, MyoG is most important in late differentiation, fusion and myotube formation [63]. Changes in the intricate regulation of SC activation, proliferation, and differentiation by triggers of muscle wasting, including inflammation, may result in impaired post-natal myogenesis, contributing to muscle atrophy. Myonuclear accretion seems to be impaired in cachexia as shown in animal studies [64]. In addition, in cachectic mice a reduced proliferation and differentiation

capability of SCs was reported, resulting in myofibers not being able to regenerate or maintain their myofiber size by myonuclear accretion leading to atrophy [65].

Evidence for a role of inflammation as a driver of cachexia and underlying mechanisms have mainly been collected in experimental models, including cultured skeletal muscle cells, animals exposed to controlled inflammatory conditions, and rodents in which diseases associated with cachexia are modeled. The marked benefit of these models is they can be deployed in experimental designs to address inflammation as a causative factor of cachexia. Moreover, they are instrumental to dissect direct and indirect effects of inflammation contributing to muscle atrophy, and provide fundamental insight in the underlying mechanisms, including the signaling pathways activated by inflammation and intra-cellular processes that cause muscle to atrophy. From these perspectives, the literature investigating the role of systemic inflammation in cachexia is discussed below.

2.1.2 Associations Between Systemic Inflammation and Cachexia in Disease Models

Various experimental disease models have provided incremental associative evidence to imply inflammation in the etiology of muscle wasting. In murine cancer cachexia models, the concurrent presence of systemic inflammation and loss of muscle and fat tissue has extensively been document. In Walker-256 tumor-bearing rats increased systemic inflammation was observed with animals

presenting elevated plasma TNF-α and IL-6 compared to non-tumor-bearing control animals [66]. These also showed increased gene expression and protein levels of MuRF1 and Atrogin-1 in hind limb muscles [66]. Similar observations were reported in an orthotopic mouse model of bladder cancer, in which increased levels of inflammatory cytokines TNF-α, IL-6, and IL-1β, and activation of pro-inflammatory pathways including NF-κB were detected in muscles of urothelial tumor-bearing animals. These observations were paralleled by downregulation of Akt- and FoxO3 phosphorylation levels, suggestive of a shift toward catabolic signaling [67]. Zhuang et al. (2016) showed that Atrogin-1 and MuRF1 levels were significantly increased in colon-26 (C26)-bearing mice suffering from cachexia, along with significant increases in circulating and muscle TNF-α and IL-6, whereas expression levels of anabolic IGF-1 were decreased [68]. A recent study by Chiappalupi et al. (2020) showed that body and muscle weight loss in mice with subcutaneously growing Lewis Lung Carcinoma (LLC) cells, was accompanied by elevated serum cytokine levels, including IL-1β, IL-6, IL-10, IFN-γ, and TNF-α. Interestingly, this study highlighted the importance of receptor for advanced glycation end-products (RAGE) in sustaining the inflammatory response in tumor-bearing mice, with RAGE null mice protected against increased systemic inflammation, body weight loss, and muscle weight loss [69].

The association between inflammation and cachexia has also been extensively explored in non-cancer, inflammatory models. Jepson et al. (1986) showed administration of LPS in fed rats resulted in inflammation and

reduced muscle weights accompanied by decreased MPS and increased proteolysis of skeletal muscle [70]. This study also showed fasted untreated animals had reduced MPS, yet fasting combined with LPS treatment exacerbated this reduction, therefore highlighting that reduced food intake alone in response to LPS was not the sole cause of skeletal muscle loss [70]. Therefore, these results show that the anorectic effects of LPS alone do not fully account for the muscle atrophy observed in this model, and therefore suggest anorexia-independent effects of inflammation in muscle wasting [70]. In line, Langen et al. (2012) showed animals with LPS-induced pulmonary and systemic inflammation exhibited more skeletal muscle atrophy than pair-fed control mice, implying an additional effect of inflammation beyond anorexia or hypophagia [71]. Similarly, LPS administered to rats to induce sepsis resulted in reduced body weights and muscle weights which could not be fully attributed to hypophagia. Here, inflammation was implicated as the primary diver of skeletal muscle loss independently of anorexia [72]. Mice with LPS and pepsin aspiration pneumonia-induced inflammation showed increased levels of IL-1β, IL-6, and MCP-1 in diaphragm and limb muscles in combination with reduced myofiber size [73]. In this model, increased levels of MuRF1 and Atrogin-1 in muscles were shown to be indicative of elevated UPS activity and calpain and caspase-3 pathway activation [73]. Langen et al. (2012) showed pulmonary inflammation by intratracheal instillation of LPS in mice resulted in a rapid increase in circulating pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and CXCL1, which preceded the loss of skeletal

muscle mass in these animals [71]. In both models of pulmonary inflammation, besides evidence for UPS-mediated proteolysis, increased Bnip3, LC3B, and Gabarapl1 expression levels were measured in skeletal muscle, suggesting elevated ALP activity in lung inflammation-induced muscle atrophy [73]. Similar findings were reported in murine models of CHF. Here cardiomyopathy, characterized by increased pro-inflammatory macrophages infiltration in cardiac muscle and elevated serum IL-6 levels resulted in elevated skeletal muscle TNF-α and CXCR4 expression and reduced fiber CSA, indicative of inflammatory myopathy [74, 75]. Furthermore, CHF was shown to reduce skeletal muscle regeneration following muscle damage [75].

Despite the diversity in primary pathology, and the degree and kinetics by which cachexia develops in these disease models, systemic inflammation and its preceding and correlation with muscle wasting appear a consistent factor beyond simple association in cachexia.

2.1.3 (Pre)clinical Evidence for Inflammation as a Cause of Cachexia in Disease

Several *in vivo* studies have highlighted the importance of specific pro-inflammatory cytokines or activation of specific inflammatory pathways in muscle wasting through genetic or pharmaceutical inhibition, showing causal evidence that inflammation is a required component of atrophy in pathological models.

In models of cancer cachexia, e.g., mice or rats with methylcholanthrene-induced sarcoma (MCG-101) or in LLC tumor bearing mice, skeletal muscle wasting

was alleviated through the blockade of the apex pro-inflammatory cytokine TNF-α through administration of anti-TNF antibodies [76, 77]. In addition, intra-muscularly inoculated LLC-tumor bearing WT mice displayed increased protein degradation and loss of muscle mass, which was prevented in transgenic littermates overexpressing the soluble TNF receptor type 1 protein (sTNF-R1) to inhibit the actions of local or circulating TNF-α [78]. While this study implies a causal contribution of TNF-α in skeletal muscle atrophy, this may result from indirect effects of TNF-α by increasing other cytokines such as IL-6 that contribute to cachexia development by impacting on skeletal muscle. Indeed, IL-6 has been implicated in cancer cachexia. $Apc^{Min/+}$ mice with cancer cachexia showed significant muscle wasting in presence of a 10-fold increase of serum IL-6 levels compared to control groups [79]. In addition, host-IL-6 was shown to be required in the development of cachexia in these mice, with $Apc^{Min/+}$/$IL\text{-}6^{-/-}$ mice showing reduced tumor burden and muscle wasting [80]. Furthermore, administration of anti-murine IL−6 receptor antibody to C−26−bearing mice attenuated muscle wasting, in support of IL-6-dependent muscle atrophy in colon cancer cachexia [81].

Administration of both anti-TNF-α and an IL1-receptor antagonists in models of cancer cachexia also shows evidence of preservation of body weight and improved food intake compared to untreated tumor-bearing controls, suggesting a common mechanism for both cytokines [82]. Furthermore, anti-TNF-α and anti-IL1-R treatment also reduced tumor growth, suggesting indirect effects of inflammation

as a determinant of tumor burden in driving atrophy [82]. Similarly, in a rat RA model, administration of soluble TNF receptor I (sTNFRI) as a TNF blocking strategy improved body weight, but also food intake compared to control groups, implying that anti-inflammatory modulation may contribute to bodyweight maintenance by blocking anorexic effects [83]. In line with that notion, in a cardiac cachexia rodent model, anti-TNF-α treatment significantly reduced losses in body and skeletal muscle mass, partly through reduced UPS activation through the attenuation of anorexia [84]. Finally, in a model of cachexia induced by *Trypanosoma cruzi*, mice treated with anti-TNF-α antibodies displayed significant attenuation of weight loss, while anti-IL-6 and anti-IFN-γ antibodies had no such effect. In addition, this protection of weight loss occurred during the acute phases of infection and was only transient in nature, suggesting early administration of anti-TNF therapies may be more effective in the early phases of cachexia [85].

The concept of a causal role of pro-inflammatory cytokines mediating cachexia in humans has only been addressed very limitedly and only in a few pathological conditions, using targeted therapeutics that deplete specific cytokines such as TNF-α and IL-6. In contrast to the experimental models, the effects of TNF-α blockade in cancer cachexia, while complicated by the diversity of disease etiologies, and limitations due to the actions of the corresponding treatments on tumor immunity, have proven less promising. Here a number of studies examining different cancer patient cohorts have failed to identify any meaningful changes in body

weights, LBM, or muscular function (determined by 6 min walk test) in response to anti-TNF-α interventions [86-89]. Studies examining anti-IL-6 in cachexia are limited. One case report in a patient with large-cell carcinoma of the lung and cancer cachexia demonstrated improved inflammatory outcomes with reduced serum IL-6 after prednisolone treatment, with no further deterioration in cachexia parameters [90]. While these studies are frequently complicated by poor accrual, recruitment, and interactions with other disease-related complications and between ongoing therapeutic interventions, thus far the causal involvement of inflammation in muscle atrophy during cancer cachexia has been difficult to assess in clinical studies.

In COPD, several TNF blocking agents have reached phase-II clinical trials, but these have been complicated by initial concerns related to increased incidence of cancer compared to the placebo control treatment arms [91], which were later contested in a long-term follow-up analysis [92]. While the original rationale for anti-TNF treatment was to intervene in the lung pathology, more recent insights suggest that specific groups of patients, in particular COPD patients with cachexia may benefit from TNF-blocking agents [92]. Studies appropriately designed to assess this remain to be initiated, however. In contrast, TNF-α blockade has proven highly effective in the management of chronic inflammatory disease such as RA [38]. It must be noted that in RA, evaluating the direct contribution of inflammation on muscle mass and function is complicated by the actions of these treatments on disease activity, which reduce pain and allow for improvements in dietary intake and physical

activity. Here, several such studies failed to report meaningful correlations in changes in body composition, in response to anti-TNF-α treatments over short durations [93, 94]. These studies are complicated by side by side comparison with disease modifying anti-rheumatic drugs such as methotrexate, which possess their own anti-inflammatory immunomodulatory effects that may mask muscle protective actions. However, studies examining anti-TNF-α intervention in RA over longer periods revealed improvement in body weight, BMI, total and fat mass relative to patients receiving standard disease management treatment [95, 96]. Moreover, promising results have been observed in alternative chronic inflammatory arthropathies and inflammatory diseases such as ankylosing spondylitis and Crohn's disease. Here, in ankylosing spondylitis, improvement in muscle strength parameters was reported following anti-TNF-α intervention, while increases in both muscle volume and strength were evident in patients with Crohn's [97, 98].

Despite the ambiguous evidence from clinical studies, the preclinical disease models strongly support a causal relationship between inflammation and cachexia. The extent to which actions of inflammation depend on interactions with other pathology-related alterations, or whether inflammation *per se* is sufficient to induce cachexia, is addressed in different experimental models described next.

2.1.4 *Causal Evidence Implying Inflammation as a Driver of Skeletal Muscle Atrophy*

Many *in vivo* studies have shown that the induction of an inflammatory state by TNF-α infusion initiate the development of cachexia, resulting in reduced food intake, loss of bodyweight, and skeletal muscle loss [99, 100]. In addition, implantation of a continuously TNF-α producing tumor cell line into mice, elicited cachexia and weight loss, with reduced food intake, compared to the control, non-secreting tumor cell line [101]. TNF-α has also shown to suppress the IGF-1 pathway and cause insulin resistance, which may also play a role in the dysregulation of macronutrient uptake and utilization [102, 103]. Mice inoculated with tumors that overexpress IFN-γ presented with severe cachexia, and IFN-γ inhibition prior to inoculation attenuated body weight loss [104], implying IFN-γ secretion rather than other tumor-dependent effects in the development of tissue depletion in this model. In mice inoculated with tumor cells expressing Fn14, a receptor for the inflammatory cytokine tumor necrosis factor-like weak inducer of apoptosis (TWEAK), significant cachexia development and reduced survival rates were observed [105]. Moreover, anti-Fn14 monoclonal antibodies prevented cachexia development, while tumor growth rate being reduced, implying local or reciprocal effects of anti-Fn14 on tumor growth and cachexia [105]. In line, chronic administration or muscle-specific transgenic overexpression of TWEAK in mice resulted in reduced body and skeletal muscle weight with an associated increased activity of UPS and NF-κB [106].

Alternatively, the release of pro-inflammatory cytokines is triggered in models of sepsis. Schakman et al. (2012) induced inflammation in rats through LPS injection, which lead to a loss of body and muscle weight [107]. IGF-1 levels were significantly reduced, and accompanied by an upregulation of FoxO1, Atrogin-1, and MuRF1. In a similar model, identical findings were reported, as well as increased muscle TNF-α expression [108], indicative of local inflammatory signaling and activation of proteolysis in this model of muscle wasting. Increased levels of systemic inflammatory cytokines may result from spill over from an inflamed site, as is the cause for pulmonary inflammation. Ceelen et al. (2018) evoked pulmonary inflammation in mice, which resulted in systemic inflammation and muscle atrophy, with accompanying of UPS-mediated proteolysis and upregulation of E3 ligases MuRF1 and Atrogin-1 [109]. Interestingly, there were no additive effects of body weight loss and muscle wasting in emphysematous mice compared to control groups after LPS exposure, and can therefore be concluded muscle atrophy was a direct consequence of the pulmonary inflammation and not affected by the presence of emphysema [110]. This group also showed evoking repetitive pulmonary inflammation in emphysematous mice, mimicking recurrent acute exacerbations in COPD, resulted in sustained muscle atrophy, which was associated with markers of impaired muscle regeneration, with altered myogenic signaling and reduced fusion capacity [64]. In line, chronic pulmonary inflammation in transgenic mice with lung-

specific overexpression of TNF resulted in muscle atrophy and an impaired muscle regenerative response compared to WT littermate control animals [111].

Taken together, these studies demonstrate inflammation is sufficient to drive muscle wasting beyond the context of disease-induced cachexia. However, these models cannot distinguish direct from indirect effects, i.e., requiring involvement of another tissue or intermediary paracrine or autocrine signal, impacting on the intramuscular processes that drive muscle mass loss in cachexia. As such understanding is instrumental for development of intervention strategies, an extensive number of studies focused on identifying the intracellular pathways responsible for sensing inflammatory signals and transducing these into atrophy responses.

2.2 Direct Effects Of Inflammation: Signaling Pathways And Activating Ligands Responsible For Relaying Direct, Muscle Atrophy-Inducing Effects Of Inflammation

The direct effects of inflammation on skeletal muscle require receptor-mediated activation of intra-muscular signaling pathways. Various signaling pathways activated by inflammatory cytokines, or inflammation-associated ligands have been implicated in muscle atrophy through regulation of muscle protein turnover or myonuclear turnover. These pathways and the corresponding activating ligands (overview in Figure 1) that have been implicated in muscle atrophy are

described in this section, including their impact on muscle protein and myonuclear turnover.

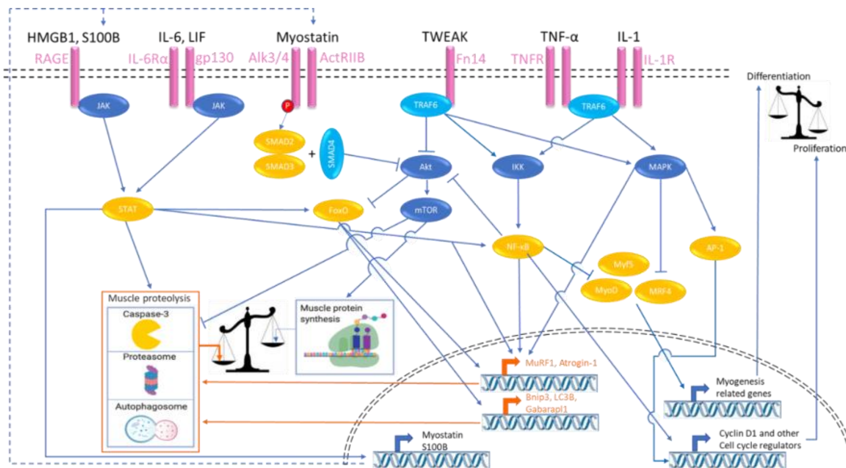

Figure 1. Signaling pathways activated by inflammatory ligands involved in cachexia-related muscle atrophy. Color refer to transcription factors (orange), proteolytic signaling (orange), kinases (dark blue), adaptor proteins (light blue), and cell surface receptors (pink). HMGB1, high mobility box 1; S100B, S100 calcium-binding protein B; RAGE, receptor for advance glycation endproducts; ActRIIB, activin receptor type IIB; TWEAK, tumor necrosis factor-like weak inducer of apoptosis; Fn14, fibroblast growth factor-inducible 14; TNFα, tumor necrosis factor-α; IL, interleukin; JAK, Janus kinase; STAT, signal transducers and activators of transcription, TRAF, TNF receptor associated factor; FoxO, Forkhead box transcription factors; mTOR, mammalian target of rapamycin; IKK, IκB kinase; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinases; JNK, c-Jun N-terminal kinase; MyoD, myoblast determination protein 1; MyoG, myogenin; MRF4, myogenic regulatory factor; AP-1, activator protein 1; MuRF-1, muscle RING-finger protein-1; Bnip3, BCL2 interacting protein 3; Gabarapl1, GABA type A receptor associated protein like 1.

2.2.1 NF-κB-Signaling

Nuclear factor-κB (NF-κB) is a TF, and activation of classical NF-κB signaling occurs in response to various inflammatory cytokines (e.g., IL-1 and TNF-α) and oxidative stress. The former act through corresponding receptor binding and activation, and recruitment of adaptor proteins, resulting in I-kappa-B kinase (IKK) activation and culminating in NF-κB nuclear translocation. TNF receptor-associated factors (TRAF) is a family of intracellular adaptor proteins that interact with the surface receptors TNFR-1 and -2, Toll-like receptor 4 (TLR4), and IL-1R. TNF-α receptor adaptor protein 6 (TRAF6) not only integrates upstream inflammatory signals, but is central to the activation of many signaling pathways including NF-κB and MAPK in response to cytokines [112, 113]. Activity if this adaptor protein is elevated in cachectic LLC-bearing mice, while TRAF6 depletion attenuated muscle wasting in tumor bearing mice [112]. Downstream, NF-κB activation has been implicated as an important step in inflammation-induced muscle wasting. Inhibition of NF−κB alleviates the cytokine-driven atrophy of muscle, thus highlighting NF-κB in the direct effects of inflammatory stimuli in muscle wasting [114]. Prevention of muscle NF-κB activation in genetically modified mice attenuated muscle wasting in a model of pulmonary inflammation-induced systemic inflammation [71]. Similarly, both, pharmacological [113] and muscle-specific genetic [115] inhibition of NF-κB prevented muscle wasting in tumor bearing mice, indicating the importance of this

TF in cancer cachexia. Conversely, muscle specific activation of the NF−κB pathway in transgenic mice resulted in profound muscle atrophy [115].

NF-κB activation has been implicated in increasing UPS proteolytic activity through the expression of E3 Ub-ligases genes Atrogin-1 and MuRF1 [52, 116, 117]. In lung inflammation-induced muscle atrophy, genetic inhibition of skeletal NF−κB inhibited the increases in MuRF1 expression [71], which was shown as a required step for muscle wasting in a similar model using MuRF1 knock-out (KO) mice [118]. In line, upregulation of MuRF1 was also required for muscle atrophy observed in response to muscle specific activation of the NF−κB pathway in transgenic mice [115]. Furthermore, NF-κB, through Akt inhibition, leads to elevated FoxO activity which stimulates the expression of UPS- and ALP-related genes, such as LC3 and Bnip3 [119]. Additionally, NF-κB prevents myoblast cell cycle exit, reduces MyoD and Myf-5 protein abundance and activity [120], and decreases MyoD mRNA expression [121], leading to impaired post-natal myogenesis [120-122]. Furthermore, *in vitro* and *in vivo* studies have also shown that serum factors from cachectic mice and patients, in an NF-κB-dependent manner, induce expression of the self-renewing factor Pax7, implying NF-kB inhibits myogenic differentiation through sustained Pax7 expression [123].

Muscle wasting-inducing properties of the inflammatory cytokines TNF-α and IL-1 have mainly been attributed to receptor-mediated activation of NF-κB, and involve increased proteolysis as well as impaired myogenesis, Several *in vitro* studies

have shown inflammatory cytokine TNF-α administration in C2C12 myocytes leads to activation of NF−κB signaling [121, 122]. The NF−κB inhibitor PDTC inhibited upregulation of MuRF1 induced by TNF-α medium *in vitro* through inhibition of NF−κB indicating the importance of this signaling pathway. TNF-α induced upregulation of the catabolic genes Atrogin-1 and MuRF1 parallel to inducing myotube atrophy in differentiated C2C12 cells [124]. In L6 and C2C12 myotubes treated with TNF-α, decreased eIF3f translation initiation factor abundance and increased Atrogin-1 levels were observed during myotube atrophy, suggestive of decreased protein synthesis and elevated proteolysis, respectively [125]. Genetic inhibition of NF-κB was also reported to prevent TNF-induced myotube atrophy [126], although in later work by this group TNF-α was postulated to act via p38 to increase Atrogin-1 and MuRF1 [124], which was confirmed in another study for TNF-α-induced Atrogin-1 in C2C12 myotubes [69]. IL-1 is an inflammatory cytokine which actions overlap with TNF-α, and can be elevated during cancer cachexia [127]. C2C12 incubation with either IL-1α or IL-1β resulted in reduced myotube size and activation of NF-κB signaling, in turn leading to increased Atrogin-1 and MuRF1 expression [128]. Another activator of NF-kB signaling implicated in muscle atrophy concerns the cytokine TWEAK and its receptor fibroblast growth factor inducible 14 (Fn14). TWEAK has been shown to be capable of inducing inflammation, which was reduced in Fn14-deficient mice, through unknown mechanisms, implying TWEAK as a feed-forward signal for an inflammatory state [129]. Following TWEAK binding to Fn14 it

can activate various signaling modules through its adaptor proteins (e.g., TRAF6), leading to NF-κB and MAPK activation [130]. Myotubes incubated with TWEAK show increased NF-κB activation [106, 131], MuRF1 and ALP-related genes such as Beclin1, and activation of caspases. Inhibition of MuRF1, autophagy, or caspase-3 blocked the TWEAK-induced degradation of MyHC and myotube atrophy [132]. Furthermore, TWEAK incubation *in vitro* can inhibit Akt phosphorylation, leading to reduced protein synthesis while stimulating protein degradation [106].

In vivo, TNF-α overexpression has been shown to impair proliferative and myogenic responses during muscle regeneration [111]. In differentiating C2C12 myocytes, activation of NF-κB by TNF-α incubation lead to the inhibition of MyoD through destabilization of MyoD mRNA [121] and MyoD protein [120]. Another study showed that when using the NF−κB inhibitor PDTC the induction of atrogenes which may have contributed to MyoD and MyHC proteolysis was inhibited in cells incubated with TNF-α [113]. TNF-α has been shown to stimulate myoblast proliferation at the expense of differentiation *in vitro* in an NF-κB-dependent manner [133]. Similarly, it was also shown IL-1 induces proliferation which was inhibited after inhibiting NF-κB, indicating this effect is NF-κB mediated [133]. Two other studies also showed IL-1 stimulated NF-κB activity *in vitro*, which also showed increases in proliferation of both primary and C2C12 myoblasts, highlighting IL-1 impacts on myogenic activity in skeletal muscle cells [122, 133]. TWEAK has also been shown to convey anti-differentiation and pro-proliferation actions through inducing

sustained NF-κB activation and MyoD degradation in addition to reduced expression levels of MyoD and MyoG *in vitro* [129, 134, 135].

Besides direct atrophy-inducing effects of TNF signaling through NF-kB, autocrine activation of parallel pathways in muscle have also been described. Treatment of TNF-α alone or combined with IFN-γ increased the expression of RAGE and its ligands S100B, and HMGB1 in C2C12 myotubes [69]. Subsequent atrophy of myotubes and increased Atrogin-1 and MuRF1 mRNA expression levels required the presence of RAGE, and involved JAK-STAT activation, implying an autocrine signaling circuit downstream of TNF-induced p38 MAPK and NF-κB activity.

2.2.2 JAK/STAT-Signaling

The JAK/STAT pathway is activated by type I (IFN-α/β), type II (IFN-γ), IL-2, and IL-6 receptor stimulation [136]. IL-6 binding to the IL-6r-Gp130 receptor complex results in the recruitment to the intracellular domain of the receptor, and subsequent activation of the JAK tyrosine kinase. After binding, JAK proteins undergo a conformational change, dimerize, and activate the STAT proteins through phosphorylation. Subsequently homo- or hetero-dimerization of STAT proteins is followed by translocation to the nucleus [137]. STAT transcriptional activation contributes to muscle wasting through various mechanisms. It stimulates CCAAT/enhancer binding protein (C/EBPδ) expression and activity, which in turn increases myostatin, MAFbx/Atrogin-1, MuRF1, and caspase-3 expression in

myofibers [31, 138, 139], enhancing muscle proteolysis. Moreover, increased myostatin expression resulting from STAT-C/EBPδ activation suppresses post-natal myogenesis [31], which in turn may negatively affect muscle mass maintenance. Furthermore, STAT was documented to regulate gene transcription by interaction with FoxO and NF-κB [140, 141].

Interleukin-6 is a pleiotropic cytokine which can induce several intra-cellular signaling pathways including JAK/STAT in a variety of cells types. Intra-cellular signaling through the binding of IL-6 to the IL-6R in turn associates with the transmembrane protein Gp130, which is ubiquitously expressed in most cells. The soluble form of IL-6R (sIL-6R) is found in most bodily fluids and also binds to IL-6, further increasing the range of target tissues for IL-6 as the IL-6-sIL-6R complex has the ability to bind and activate to Gp130 on any cell, this is known as “trans-signaling” [142]. IL-6 has been implicated as a core mediator of cancer cachexia. Indeed, systemic IL-6 concentrations increase with intestinal tumor development in $Apc^{Min/+}$ mice and is associated with elevated p-STAT-3 and Atrogin-1 mRNA levels [79]. In addition, host-IL-6 was shown to be required in the development of cachexia in these mice, with $Apc^{Min/+}$/IL-$6^{-/-}$ mice showing reduced tumor burden and muscle wasting [80]. In line, increased circulating IL-6 levels and elevated STAT-3 signaling were detected in skeletal muscle of C26 cachectic mice, and inhibition of STAT-3 attenuated muscle atrophy *in vitro* and *in vivo* [143]. Blockade of IL-6R through administration of an anti-murine IL-6R antibody in C26-bearing mice also showed

attenuated muscle loss and reduced expression of cathepsin B and L in muscle compared to tumor-bearing controls [81], highlighting the requirement of IL-6 and IL-6 signaling in this experimental model of cancer cachexia. Conversely, overexpression of human IL-6 increased expression of proteasomal subunits cathepsins B and L in muscle and induced muscle atrophy in transgenic mice [144], highlighting that chronic elevation of circulating IL-6 is sufficient to cause muscle wasting. In support of a role for increased proteolysis, intra-peritoneal injections of IL-6 lead to increased muscle atrophy in rats measured by tyrosine and 3-methylhistidine release [145]. In addition, reduced phosphorylation of PS6K1, indicative of reduced translational capacity, and protein synthesis have been reported in muscles infused with IL-6 [138]. Local IL-6 infusion into the TA muscle decreased total and myofibrillar protein content in rats [138], suggesting that atrophy-inducing effects of IL-6 are the result of direct actions of IL-6 on skeletal muscle. In support of this, C2C12 myotubes treated with recombinant IL-6 did show reduced myotube diameter, reduced mTOR and 4EBP-1 phosphorylation, and increased STAT3 phosphorylation and Atrogin-1 transcription, showing IL-6 suppresses mTOR and therefore reducing protein synthesis, in addition to increasing atrogene expression [146]. However, other studies have shown little effect of IL-6 on skeletal muscle both *in vivo* and *in vitro* [147, 148], which may be explained by differences in IL-6 levels, shorter exposure regiments, or the pleiotropic nature of IL-6 in skeletal muscle, e.g., the source of IL-6 [149].

Gp130 has been implicated as the main cellular receptor in skeletal muscle to mediate the IL-6 effects in cancer cachexia. Mice injected with LLC with a genetic deletion of Gp130 specifically in skeletal muscle showed attenuated muscle wasting compared to WT controls, primarily through reduced STAT signaling and atrogin-1 and FoxO3 activation [150]. In line, hyperactivation of STAT3 signaling through Gp130 activation in gp130$^{F/F}$ knock-in mice with a k-Ras-driven lung carcinoma developed cachexia with reduced muscle and fat mass and reduced life expectancy compared to k-Ras mice without Gp130 hyperactivation [151]. Although these results show the importance of the activation of Gp130 and STAT signaling in cancer cachexia, Gp130 activation is not solely restricted to IL-6.

Leukemia inhibitory factor (LIF) has also recently been identified as a cytokine, which activates the same receptor as IL-6 and also mediates skeletal muscle atrophy through STAT and ERK signaling [152]. Seto et al. showed in a murine model of C26 colon carcinoma with muscle atrophy, serum levels of LIF increased in parallel to tumor development [152]. LIF was actively secreted by C26 tumor cells, whereas TNF-α and IL-6 were not, and incubation of C2C12 myotubes with LIF was sufficient to induce atrophy. Conversely, LIF inhibition in tumor cell conditioned media (CM) prevented CM-induced myotube atrophy *in vitro*, while genetic inactivation of STAT3 in myofibers was sufficient to suppress atrophy *in vivo* [152]. RAGE is part of the immunoglobulin superfamily and known as a key mediator of several pathological processes. It is activated by ligands including high mobility group

box 1 (HMGB1) and the S100 calcium−binding protein B (S100B), which are secreted by various cell types, including tumor cells and damaged myofibers [69]. Furthermore, inflammatory cytokines activate a feed-forward RAGE signaling loop by inducing HMGB1, S100B, and RAGE expression in skeletal muscles [69]. Its potential relevance to cancer cachexia is suggested by increased serum levels of S100B and HMGB1 in the serum of cancer patients [69, 153], and highlighted by the observation that LLC-bearing RAGE/KO mice displayed delayed body and muscle weight loss, reduced Atrogin-1 and MuRF1 expression levels, and prolonged survival time compared to WT mice. The cytosolic domain of RAGE connects to JAK/STAT3 signaling, implicated in increased protein degradation and decreased differentiation, but has also been reported to activate the tyrosine kinase protein, Src, which is implicated in several other downstream signaling hubs, such as ERK1/2, p38 MAPK, JNK, and NF-κB [154].

2.2.3 MAPK Signaling

The MAPK pathway controls growth and stress responses in a myriad of cell types, including skeletal muscle. MAPK signaling is activated by cellular stress, growth factors, and pro-inflammatory cytokines (e.g., IL-1 and TNF-α) [155]. The MAPK family of proteins consists of four distinct signaling pathways, namely, extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK, c-Jun NH2-terminal kinases (JNK), and ERK5 [156]. p38 MAPK mediates upregulation of MuRF1

and Atrogin-1 in response to TNF-α by an unknown mechanism [69, 124]. IL-1 signaling has also been shown to stimulate phosphorylation of p38 MAPK, leading to increased atrogin-1 expression, independent of Akt/FoxO signaling [128]. Furthermore, p38 phosphorylates MRF4, thus inhibiting the expression of selective myogenic genes in late myogenesis, and antagonizes the JNK proliferation-promoting pathway [157, 158]. JNK mediates AP-1 activation, which is a signaling molecule that controls proliferation and differentiation through transcriptional regulation of cell-cycle regulators such as cyclin D1, cyclin A, and cyclin E [159, 160], and has been implicated in muscle atrophy responses [126]. When treated with TNF-α, C2C12 increased p-ERK in differentiating myoblasts, which correlated with suppressed MyoD and MyoG levels, and reduced accretion of myosin heavy chain content. Administration of the ERK inhibitor PD98059 to C2C12 cells prevented this inhibitory effect of TNF-α on myogenic differentiation [161].

2.2.4 SMAD-Signaling

The smad pathway is activated by multiple ligands, but in the context of muscle mass control, Myostatin/GDF8, a member of the transforming growth factor-β (TGF-β) family, is the best described [162], next to GDF11 [163] and Activin-A [164]. Myostatin is a myokine, and its autocrine and paracrine effects act as a break on skeletal muscle growth. Myostatin has been found to be associated with cancer cachexia and its expression is stimulated through the JAK/STAT pathway [31, 165].

This positions Smad signaling secondary to transcriptional activation of Mstn by inflammatory cues. Binding of myostatin to ActRIIB results in the phosphorylation of Smad2/3 [166] and activation of Smad signaling, which reduces p-Akt levels [167], consequently activating caspase-3 and FoxO, and resulting in increased protein degradation [31, 52]. Accordingly, downregulation of p-Akt and p-FoxO3 accompanied by myostatin and activin A overproduction in the muscle were seen in mice with bladder cancer [67], implying myostatin involvement in cachexia triggered by various cancer types [67, 168]. Myostatin administration is sufficient to induce cachexia in mice through ActRIIB signaling [165]. Conversely, blockade of the ActRIIB receptor prevented cachexia in C26 tumor bearing mice, without affecting increased circulating levels of IL-6, TNF-α, and IL-1β [169], implying ActRIIB signaling acts independent, or downstream of inflammation-associated muscle atrophy through autocrine expression of ActRIIB activating ligands like myostatin or Activin-A [164]. In support of this notion, inhibition of myoblast differentiation by inflammatory cytokines was found to require *de novo* Activin-A production [164], implying smad signaling secondary to an autocrine mechanism activated by inflammation.

Combined, these studies identify a myriad of inflammatory cytokines and ligands as mediators of inflammation, which directly impact on skeletal muscle through receptor-mediated signaling which affects muscle protein turnover in favor of proteolysis or impairs myogenesis, ultimately resulting in muscle wasting.

2.3 Indirect Effects of Inflammation

In addition to the direct effects inflammatory cytokines induced by receptor-mediated activation of signaling pathways in skeletal muscle, cytokines also cause dysregulation of other tissue and organ systems which indirectly contribute to muscle wasting and cachexia development. As such, dysregulation of the hypothalamus-pituitary-adrenal (HPA) axis and adrenocorticoids, anorexia and malnutrition, changes in adipocyte behavior, and hepatic metabolism have shown to impact cachexia progression (see overview in Figure 2).

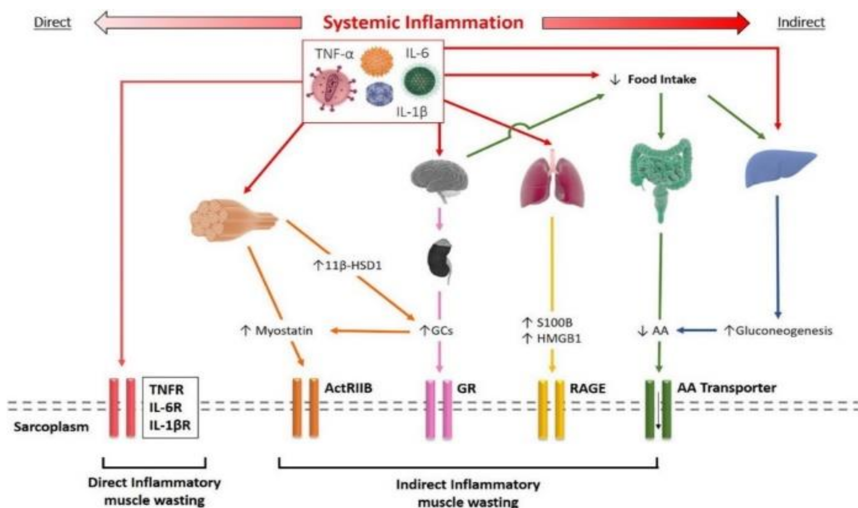

Figure 2. Schematic representation of the direct and indirect effects of systemic inflammation resulting in muscle wasting. Inflammatory cytokines such as TNF-α and IL-1β can bind to their receptors on the sarcolemma driving skeletal muscle wasting directly. Alternatively, cytokines may elicit their effects indirectly through several mechanisms, including increased myostatin and glucocorticoid signaling, release of S100B and HMGB1 by inflamed tissue, or reduced amino acid (AA) availability.

2.3.1 *Involvement of the HPA Axis and Corticosteroid Synthesis in Cachexia*

Glucocorticoids such as cortisol are endogenous pleotropic hormones that play an essential role in glucose mobilization and energy metabolism, as well as having potent anti-inflammatory immune modulatory actions [170]. In the 1950s, Kendall, Reichstein and Hench received a Nobel prize for their work in the isolation and application of the GCs in the treatment of chronic inflammatory diseases such as RA. Unfortunately, the clinical efficacy of endogenous and synthetically derived GCs was tempered by severe metabolic side effects, including osteoporosis, truncal obesity, and muscle wasting. The mechanisms underpinning GC induced muscle wasting are compound, with evidence of reduced anabolic IGF-1 signaling and increased anti-anabolic myostatin production, resulting in a reduction in the mTOR signaling pathway, as well as induction of catabolic UPS and autophagy mediated muscle protein degradation, secondary to increased FOXO1 signaling [171-173], and decreased myogenesis [174].

Due to their muscle atrophy-inducing actions, considerable interest exists regarding possible dysregulation of endogenous steroid synthesis in conditions such as chronic inflammation and cancer cachexia. In particular, the HPA axis is a central regulatory target activated in response to systemic inflammation and stress that has been widely investigated [175, 176]. This critical homeostatic regulatory pathway mediates the synthesis and release of the endogenous GC hormone, cortisol, from the zona fasciculata of the adrenal cortex [177]. Classically, the HPA axis is under

central circadian regulation by the hypothalamus, where it drives a pulsatile diurnal secretion of cortisol through the release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus. This in turn results in the systemic release of adrenocorticotropin (ACTH) from the anterior pituitary, which binds to the MC2R receptor in the adrenal cortex to initiate adrenal cortisol synthesis and release [177, 178]. During inflammation, pro-inflammatory factors such as TNF-α, IL-1β, and IL-6 can act at all levels of the HPA axis to increase CRH, ACTH from the hypothalamus and pituitary, and cortisol synthesis and release from the adrenals [179-181]. Conversely, negative regulation of this pathway is achieved through the direct suppression of CRF and ACTH by cortisol.

In particular, focus has fallen upon the concept that either inflammatory cytokines, or disease treatments (such as chemotherapy in cancer) [182], cause dysregulation of central hypothalamic/pituitary negative feedback resulting in the over activation of the HPA axis in cachexia [183], leading to circulating steroid excess and GC induced muscle wasting as well as loss of adipose tissue [184, 185]. In addition, the mixed immunomodulatory actions that circulating GCs may mediate in cancer immunity and in suppression of pro-inflammatory cytokines may also influence disease progression and cachexia. However, studies exploring the dysregulation of the HPA axis specifically in cachexia have yielded mixed results. In mice, several studies report increased activity of the HPA axis in models of cancer cachexia and COPD, coinciding with weight loss [185-188]. de Theije et al.

(2018) showed that hypoxia-induced muscle wasting in a murine model of COPD was in turn dependant on GC signaling with GC receptor/KO mice being partly protected from muscle wasting [188]. Several studies in cancer patients (including colorectal, prostate, and breast) report dysregulation of the HPA axis with increased levels of serum cortisol [187, 189-191]. In several of these instances, increases in serum GCs were linked with elevated levels of the pro-inflammatory cytokine IL-6, suggesting this may be a key mediator of increased HPA axis activity. However, whether these changes regulated cachexia in addition to influencing tumor immunity was not fully elucidated [187]. In addition, muscle GC signaling was found to be required for cancer-induced cachexia [192], and muscle atrophy in response to inflammation-evoking cytotoxic chemotherapy was shown to depend on intact GC signaling in skeletal muscle [182]. Similarly, muscle-specific deletion of GR prevented endotoxin-induced muscle atrophy [192]. In line, LPS injections in rats induced an inflammatory response, body weight loss, and muscle wasting including upregulation of FoxO and other atrogenes [107]. In contrast to TNF-α and NF−κB inhibitors, only inhibition of the GC receptor using RU-486 blunted LPS-induced atrogene expression in this model, highlighting the importance increased GC signaling in inflammation-associated muscle wasting [107]. Consequently, the true nature of HPA axis and steroid dysregulation in cachexia, while of significant interest, have proven hard to fully elucidate and show significant disease specific variation.

2.3.2 *A Role for Pre-receptor Steroid Metabolism in Glucocorticoid-Induced Muscle Atrophy*

While the systemic regulation of circulating endogenous GC levels is determined through the HPA axis, peripheral exposure to GCs is mediated through their tissue specific pre-receptor metabolism. This is primarily mediated by the 11beta-hydroxysteroid dehydrogenase (11β-HSD) enzymes types 1 and 2 [193]. Here, 11β-HSD1 primarily mediates the peripheral conversion of the inactive GC precursor cortisone, to its active counterpart cortisol (11-DHC to corticosterone in rodents) within target tissues, where it greatly amplifies local GC signaling. In contrast, 11β-HSD2 solely inactivates GCs, converting active cortisol to cortisone (corticosterone to 11-DHC in rodents) blocking local GC signaling. 11β-HSD1 shows a diverse pattern of expression across a wide array of tissues including liver, fat, muscle, bone, and in immune cells [194-196]. In contrast, 11β-HSD2 expression appears to be limited to tissues such as the kidney where it protects against inappropriate activation of the mineralocorticoid receptor by GCs [197]. Renewed interest in the roles of 11β-HSD1 in inflammatory muscle wasting and cachexia have been fueled by observations that its expression and GC activation are potently upregulated in peripheral tissues such as muscle in response to pro-inflammatory factors such as IL-1β and TNF-α [196, 198]. These studies raised the possibility that under conditions of chronic inflammation, local amplification of GC signaling by 11β-HSD1 may represent a critical component in mediating inflammatory muscle

wasting. This concept was lent further credit following a seminal study by Morgan et al. (2014) demonstrating that the systemic transgenic deletion of 11β-HSD1 in murine models of GC excess completely abrogated GC induced muscle wasting [199]. However, the only study to examine the role of inflammatory 11β-HSD1 in muscle revealed a complex interplay between the anti-inflammatory actions of GCs versus their anti-anabolic catabolic actions [196]. Here, while the transgenic deletion of 11β-HSD1 in murine models of inflammatory polyarthritis resulted in reduced GC signaling in muscle, the exacerbation of muscle inflammation drove a more florid muscle wasting phenotype. Consequently, the role of 11β-HSD1 in other forms of muscle wasting and cachexia requires further investigation.

2.3.3 Inflammation-Driven Anorexia and Muscle Wasting in Cachexia

Cachexia development is profoundly impacted by the accompaniment of anorexia, categorized by reduced appetite and nutritional deficit which ultimately leads to catabolism of lean body and adipose tissue [200]. Anorexia–cachexia is distinct from starvation, where skeletal muscle loss is less apparent compared to adipose tissue. Adipose tissue is a reservoir for energy, and therefore in times of starvation or reduced energy intake, catabolism of adipose tissue allows the release of energy which is then used in processes that maintain skeletal muscle mass, however, in cachectic patients both muscle and fat tissue are catabolized as energy sources. In addition, nutritional interventions alone are unable to reverse or alleviate

this catabolic phenotype [201-203]. Although the pathogenesis of anorexia–cachexia is multifactorial, inflammatory cytokines have been shown to be implicated in the development of anorexia in cachectic patients through an amalgamation of mechanisms [204]. In some disease states, such as cancer, tumor burden has been implicated in driving anorexia–cachexia through dysphagia or dysregulation of gastro-intestinal function, ultimately leading to reduced food intake and nutritional deficit [205].

Rats receiving a single dose of human TNF-α resulted in increased muscle proteolysis and anorexia [206, 207], while tumor bearing rats receiving TNF-α inhibitors had markedly improved nutritional intake and body weights [77]. These findings suggest TNF-α indeed plays a pivotal role in inducing anorexia, although its full contribution the development of cachexia is yet to be elucidated. Plata-Salamán et al. (1988) demonstrated TNF-α administration in rodents suppressed food intake in a dose-dependent manner, through the cytokine directly acting on glucose-sensitive neurons in the central nervous system (CNS) to suppress appetite [208]. Lung cancer patients exhibiting anorexia showed reduced hypothalamic activity compared to non-anorexic patients; however, circulating levels of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1 were not significantly different between groups [209]. Another cytokine implicated in anorexia–cachexia is IL-1, with several studies observing its effects on food intake. These anorexia-inducing effects of IL-1 have been illustrated in several *in vivo* studies, with both peripheral and central

administration decreasing food intake in rodents [210]. Furthermore, Layé et al. (2000) previously showed increases in IL-1 in the hypothalamus in rodents upon LPS administration and reduced food intake, and IL-1 antagonists preventing LPS-induced anorexia [211]. However, many of these *in vivo* studies also show development for tolerance to cytokines, and therefore the interpretation of results is often debated [212]. Another study highlights the importance of central regulation of appetite in response to inflammation, with GLP-1 receptor antagonist mitigating anorexia induced by LPS in rats [213].

Peripheral hormones that directly affect nutrition status through central actions controlling appetite have also been shown to play an important role in anorexia–cachexia. Ghrelin, a peptide released in the gut shown to stimulate appetite, is decreased in response to acute inflammation [214, 215]. In contrast, chronic inflammation in animal models and cachectic patients present with increased ghrelin levels [216-218], possibly a compensatory effect of ghrelin resistance in cachectic and catabolic states [219]. Mechanisms underpinning ghrelin's role in anorexia–cachexia have not yet been established; however, experimental models of cachexia have demonstrated ghrelin administration suppressed weight loss and alleviated skeletal muscle wasting through increased food intake [220, 221]. Another cytokine shown to be of importance in the role of developing cachexia–anorexia is leptin, which is released from adipocytes and signals to the hypothalamus to regulate nutritional intake as a satiety cue. Leptin has been

shown to be increased in rodents and humans exhibiting cachexia in many disease states, such as CHF [222]. Therefore, the role of leptin in anorexia–cachexia is not well established and is often speculated that the alterations in leptin levels may be in response to malnutrition and reduced fat mass rather than a consequence of elevated inflammatory cytokines.

While these data collectively support a role for pro-inflammatory cytokines in the development of anorexia, due to cachexia's multifactorial phenotype, it is difficult to underpin the mechanisms in which cytokines may drive anorexia–cachexia. However, a plethora of research indicates that energy deficits in combination with reduced hypothalamic response may play a pivotal role in anorexia–cachexia (Ramos et al., 2004).

2.3.4 Role of Inflammation-Induced Alterations in Adipocyte and Hepatic Metabolism in Cachexia

There is increasing evidence to show preserving adipose tissue in cachexia can improve mortality and quality of life [223]. Lipolysis stimulation during cachexia can be induced by anorexia; however, there is also evidence for inflammation-induced lipolysis. Reduction in food intake or starvation induces lipolysis to release energy stores; however, as lipid stores are depleted, other catabolism of tissues will ensue to provide sufficient energy, of which a main energy source is amino acids derived from skeletal muscle proteolysis [224]. As discussed previously,

inflammation can induce anorexia–cachexia, reducing food and energy intake, which ultimately leads to the reduction of fat mass and loss of white adipose tissue (WAT) through lipolysis. Cachexia is also associated with loss of skeletal muscle and WAT through increased energy expenditure [225], and related to increased inflammation in pancreatic cancer patients presenting with elevated resting energy expenditure in addition to increased CRP levels [226]. Patients with cancer cachexia were shown to have increased levels of circulating IL-6 and enhanced lipolysis compared to weight-stable cancer patients, which was not attributed to enhanced locally expressed IL-6 levels, implicating not the inflammatory infiltrate but other triggers for adipose tissue wasting [227]. One mechanism that has been described to contribute to this increased energy expenditure is the remodeling of WAT into brown adipocytes, which has been suggested to occur prior to skeletal muscle wasting in cancer-cachexia [228]. This increase in brown adipose tissue increases thermogenesis in these patients [229], which ultimately leads to an increased requirement for energy, and thus increased energy expenditure. Interestingly, IL-6 has been implemented in increasing uncoupling protein 1 (UCP1) expression, a protein found in brown adipose tissue that increase thermogenesis [230]. In addition, mice with syngeneic grafts of C26 cells lacking IL-6 showed protection against weight loss and reduced UCP1 expression compared to mice with active IL-6 C26 cells, thus highlighting the importance of this cytokine in WAT browning, and therefore increased energy

expenditure in cachexia [228]. However, the exact role of WAT browning in skeletal muscle loss in cachexia requires further investigation.

Although various studies have shown the effects of inflammatory cytokines on adipose tissue and skeletal muscle, only few address these in the context of cachexia. Mice s.c. injected with LLC or B16 melanoma cells showed cachexia development, with reduced body weights, WAT loss, muscle wasting, and increased serum TNF-α and IL-6 levels [231]. Inhibition of lipolysis through genetic deletion of adipose triglyceride lipase (Atgl), a mediator of lipolysis, in tumor-bearing animals showed protection against cachexia development, with reduced WAT and skeletal muscle loss. However, TNF-α and IL-6 levels remained increased in the serum of these animals, highlighting a possible indirect action of these cytokines in driving cachexia and adipose tissue loss through lipolytic mechanisms [231]. Importantly, this study also emphasizes the importance of crosstalk between adipose tissue and skeletal muscle, as inhibition of lipolysis resulted in reduced skeletal muscle wasting, therefore suggesting that altered free fatty acid or adipokine release may play a role in skeletal muscle wasting. Adipose tissue secretes adipokines, such as leptin, with endocrine functions including satiety and whole-body metabolism [232]. Several inflammatory cytokines such as IL-6, TNF-α, and IL-1β are adipokines as well as myokines, and have been implicated in reciprocal control of adipose and muscle mass [233, 234] and metabolism [235]. Further research, however, is required to

disentangle the role of inflammation in adipose and muscle reciprocal effects in the context of cachexia.

Although the liver is a central regulator of metabolism, there is relatively little research examining a role of the liver in the association between inflammation and cachexia, which is surprisingly considering the liver is the major site for muscle proteolysis-derived amino acids for utilization in gluconeogenesis and acute-phase protein synthesis, such as CRP [236], and elevated CRP levels are the most frequently applied additional criteria to assess cachexia [237]. Indeed, profound hepatic alterations are observed prior to and during the progression of cancer cachexia, including alterations in fat metabolism, collagen deposition, and fibrosis [238]. In line, despite not evaluating liver-anatomical changes, alterations in liver metabolism in inflammation associated cachexia have been reported. Apc$^{min/+}$ mice with severe cachexia were shown to have increased levels of acute phase protein haptoglobin, revealing hepatic alterations in inflammation-associated cancer cachexia [239]. In a model of pancreatic cancer cachexia, inhibition of proliferator-activated receptor-alpha (PPAR-α) through IL-6 resulted in hypoketonemia and subsequent activation of the HPA axis, ultimately leading to increased GC release and enhanced muscle proteolysis [187]. In addition, Goncalves et al. showed adult *Kras*$^{G12D/+}$*;Lkbl*$^{f/f}$ (KL) mice with lung cancer and cachexia presented with increased IL-6 levels, increased gluconeogenesis in the liver, reduced hepatic fatty acid oxidation, and hypoketonemia. Skeletal muscle atrogenes MuRF1 and Atrogin-1 were upregulated

and also noted a decrease in type II fiber CSA [240]. PPAR-α inhibitor fenofibrate restored hepatic ketogenesis, which in turn reduced the requirement for the liver to use gluconeogenesis and alleviate the need for type II skeletal muscle degradation for amino acids [240]. These results therefore show the indirect effects of both liver metabolism and GCs on skeletal muscle wasting in cachexia.

2.3.5 *Myokines as a Nexus and Opportunity in Modulating Inflammation-Associated Cachexia*

Apart from the participation of other tissues as an intermediary step between inflammation and induction of muscle wasting, a role for autocrine, paracrine, and even endocrine acting signals derived from skeletal muscle in cachexia is emerging. These concern the "myokines," e.g., cytokines, growth factors, and other peptide-based molecules released from skeletal muscle [241]. In the context of muscle mass regulation, Mstn is a well-characterized myokine for its muscle growth-inhibitory actions. Increased expression [242] and signaling [31] of Mstn in skeletal muscle may constitute an autocrine mechanism of Mstn-dependent muscle wasting in response to inflammatory cues. In line with this notion, blockade of the ActRIIB receptor to inhibit Mstn signaling prevented cachexia in C26 tumor bearing mice, without affecting increased circulating levels of IL-6, TNF-α, and IL-1β [169]. Interestingly, increases in muscle Mstn expression and secretion may also contribute to muscle wasting in an endocrine route in case of RA, as Mstn has been

implied in inflammatory bone destruction, aggravating RA-associated muscle loss [243]. In C26 tumor-bearing BALB/c mice, involvement of myokines was further indicated as increased muscle IL-6, IL-6R, and myostatin expression accompanied muscle wasting in these mice [244]. Recent work has revealed GDF15 as a myokine, which is expressed at low levels during homeostasis, but can be induced by muscle contraction [245], or metabolic stress [246] or increased GDF11 levels [247] in skeletal muscle. GDF15 circulating levels correlate inversely with skeletal muscle mass in COPD [248], and increasing GDF15 levels are sufficient to induce dramatic weight loss [249]. As thus far no evidence supports the expression of the GDF15 receptor, GFRAL, a co-receptor of the Ret tyrosine kinase, in skeletal muscle, this suggests endocrine effects of GDF15 when secreted as a myokine. As such, muscle derived GDF15 was reported to stimulate lipolysis in adipocytes [245], which in the context of cachexia could contribute to adipose tissue depletion. Importantly, elevation of GDF15 suppresses appetite via activation of hypothalamic neurons [249], and the cachexia-inducing properties GDF15 are thought to be a result of anorexia [249]. Although induction of GDF15 expression by TNF-α and NF-κB regulation has been shown for other cell types [250], it remains to be explored whether GDF15 expression increases in skeletal muscle in response to inflammatory cytokines.

In contrast, other myokines including IL-15 [251] and musculin [252] have been attributed anabolic effects or anti-catabolic effects on skeletal muscle, at least

in part mediated through autocrine mechanisms. In addition, IL-6, when secreted by skeletal muscle in response to stimuli such as exercise, exerts endocrine effects such as lipid oxidation [253], which contribute to organismal homeostasis. Interestingly, in tumor-bearing mice, exercise was found to attenuate tumor growth, which correlated with increased IL-6 levels post-exercise, and systemic IL-6 blocking experiments revealed IL-6 may actually contribute to hindering tumor growth [254]. Moreover, exercise-induced increases in IL−6 contribute to an anti-inflammatory systemic environment, by increasing the production of the anti-inflammatory cytokines, IL−1β receptor antagonist (IL−1ra), and IL−10 [255]. A recent murine study suggested that IL-6 may induce either pro- or anti-inflammatory actions depending on cell source [256], potentially explaining the beneficial, suppressive effects on tumor growth and immunomodulatory actions of muscle derived IL-6. In addition, C2C12 differentiating myoblasts showed increased IL-6 levels during differentiation in combination with increased STAT3 phosphorylation. Blockade of IL-6 independently showed reduced differentiation of myotubes, highlighting the crucial role IL-6 has in differentiating myotubes [257]. As such, these studies may reflect an endocrine cachexia-modulating potential of myokines.

2.4 Conclusion And Future Perspectives

Despite the overwhelming preclinical evidence to imply inflammation as both sufficient and required in disease-associated cachexia, this has not translated

into unambiguous success of cytokine-depleting therapeutic agents to reverse cachexia in patients with cancer or chronic disease. This may reflect the complex interactions within an inflammatory response, rendering a therapy based on inhibition of a single cytokine therapy insufficient. Combined blocking approaches [85], or downstream inhibition of molecules at which inflammatory cues convergence, like NF-κB [113] or STAT3 [198] have shown effective in experimental models and warrant further exploration for pharmacological modulation. In addition, the timing of anti-inflammatory treatment may be of key importance. Much of the evidence in the experimental models indicates inflammation precedes a cachectic phenotype, and anti-inflammatory interventions successfully modulating cachexia in preclinical studies are without exception started prior to initiation of cachexia development. Consequently, for anti-inflammatory agents to be effective in a clinical setting, this may require interventions to start in patients at risk for cachexia, i.e., “pre-cachectic,” for subsequent evaluation of their ability to prevent or delay onset of cachexia. Bearing time in mind as an important determinant of the efficacy anti-inflammatory modulation, its downstream signaling should be further considered. Feed-forward signals that transform inflammatory cues of systemic origin into an autocrine, muscle atrophy-promoting signal, have been reported for Mstn [31] and Activin-A [164]. Receptor blocking agents for these ligands are available, and the first clinical trials have yielded promising results in terms of safety and efficacy in COPD patients with low muscularity [258]. These ActRIIB inhibitors

are continuously refined [259], and further improvement in their efficacy to halt or even reverse cachexia progression is anticipated when provided as an integral part of a multimodal therapy, i.e., combined with appropriate nutritional support and tailored exercise programs. Similarly, desensitizing skeletal muscle to the atrophy-inducing effects of GCs, by inhibition of local GC-activation using therapeutic 11β-HSD1 inhibitors that are currently in clinical trials for other applications [260], may be a route to explore the potential of blocking the indirect effects by which inflammation contributes to muscle wasting. Finally, skeletal muscle tissue itself may hold the key to counteracting inflammation driven cachexia, as myokines have been attributed very potent immunomodulatory features, which in future research deserve further investigation in their potential to prevent and reverse skeletal muscle wasting in cachexia.

Combined, the extensive efforts to delineate the underlying mechanisms of inflammation-associated cachexia have revealed insights that provide multiple leads to evaluate novel, more selectively targeted therapeutic approaches in this debilitating condition.

Author Contributions

JW and LK systematically reviewed potentially relevant manuscripts to extract and synthesize the findings into the various sections of the review. RL conceived the focus of the manuscript. RH and RL defined the structure of the review, critically edited the content of the paragraphs, and defined conclusions. All authors contributed to the article and approved the submitted version.

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Conflict Of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 3 - 11β-HSD1 determines the extent of muscle atrophy in a model of acute exacerbation of COPD

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Abstract

Muscle atrophy is an extra-pulmonary complication of acute exacerbations (AE) in chronic obstructive pulmonary disease (COPD). The enzyme 11 beta-hydroxysteroid dehydrogenase 1 (11β-HSD1) activates glucocorticoids (GCs) within muscle and contributes towards GC-induced muscle wasting. We examined the contribution of 11β-HSD1 in inflammatory muscle wasting using a murine model of AE-COPD in animals with transgenic global deletion of 11β-HSD1. WT and 11βHSD1/KO mice received two intra-tracheal (IT) instillations of elastase to induce emphysema (COPD), followed by a single bolus of vehicle or IT-LPS to mimic AE. After 48 hours muscle, serum and lung tissues were collected. µCT scans were collected prior and following IT-LPS, to assess emphysema development and muscle mass changes, respectively. Serum cytokine and GC profiles were determined by ELISA. *In vitro* myonuclear accretion and cellular response to serum and GCs were determined in C2C12 and human primary myotubes. Muscle wasting was exacerbated in LPS-11βHSD1/KO animals compared to WT controls. RT-qPCR and western blot analysis showed elevated catabolic pathways (Atrogin-1, MuRF-1) in LPS-11βHSD1/KO animals relative to WTs and suppressed anabolic and anti-catabolic pathways (p-S6(S235/236), p-FoxO1(S256)). Serum corticosterone levels were higher in LPS-11βHSD1/KO animals, whilst C2C12 myotubes treated with LPS-11βHSD1/KO plasma or exogenous GCs had a reduced myonuclear accretion capacity relative to WT counterparts. This study reveals that 11β-HSD1 protects against muscle wasting in a

model of AE-COPD. Its transgenic deletion results in circulating corticosteroid dysregulation, favouring increased catabolic and decreased anabolic muscle metabolism.

Keywords: 11beta-hydroxysteroid dehydrogenase type 1, muscle atrophy, inflammation, COPD, glucocorticoids

3.1 Introduction

Chronic obstructive pulmonary disease (COPD), characterised by remodelling of the airways (bronchitis) and destruction of the lung parenchyma (emphysema), is predicted to be the third leading cause of death worldwide by 2030 [1]. Skeletal muscle wasting is a severe extra-pulmonary complication of COPD, particularly in patients with emphysema [2, 3], which contributes to frailty and poor disease outcomes, and is an independent predictor of mortality [4]. To date, therapeutic strategies designed to prevent or reverse the acute process of muscle wasting in COPD patients are limited, and primarily restricted to exercise and nutritional intervention, with limited efficacy in the acute exacerbation disease phase [5, 6].

Acute exacerbations of COPD (AE-COPD), typically secondary to acute pulmonary and systemic inflammation with infection, accelerate muscle loss in COPD [7]. Glucocorticoids (GCs), such as cortisol (corticosterone in mice), are a class of endogenous anti-inflammatory steroids that are elevated in response to systemic inflammation [8]. This increase in circulating cortisol is mediated by the inflammatory activation of the hypothalamic-pituitary-adrenal axis (HPA) resulting in increased adrenal output [8]. Synthetic therapeutic glucocorticoids possess potent anti-inflammatory immunomodulatory properties and are frequently utilised in the management of COPD patients during inflammatory exacerbation [9]. Both elevated endogenous and therapeutic GCs are associated with increased GC signalling within

skeletal muscle [10], and contribute to muscle atrophy in human disease, through the activation of proteolytic pathways, and suppression of anabolic signalling and myonuclear turnover [11-13].

The enzyme 11 beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) converts inactive endogenous GCs precursors (cortisone in human, 11-dehydrocorticosterone in mice) into their active form (cortisol and corticosterone respectively), where it amplifies GC signalling and determines peripheral tissue exposure to GCs [14]. This enzymes expression is potently upregulated in muscle in response to pro-inflammatory mediators present during acute exacerbation of COPD (including cytokines such as IL-1β and TNF-α), as well as by alternative factors implicated in COPD-associated muscle wasting, such as hypoxia, fasting and therapeutic corticosteroids [15, 16]. Several studies have revealed a critical role for 11β-HSD1 in determining local endogenous and therapeutic GC levels within muscle, which in turn modulate anti-anabolic and catabolic muscle metabolism in primary culture, *ex vivo* and *in vivo* models [15, 16]. However, whilst therapeutic inhibitors of 11β-HSD1 have been widely explored in phase II clinical trials in the management of metabolic diseases such as hypertension, osteoporosis and insulin resistance, their application in the management of muscle wasting in inflammatory diseases such as COPD remain poorly defined [17-24].

The objective of this study was to ascertain the contribution of endogenous corticosteroid activation and amplification by 11β-HSD1 towards the

pathophysiology of skeletal muscle wasting during an acute exacerbation of emphysematous-COPD and test the hypothesis that therapeutic 11β-HSD1 inhibition can prevent GC induced muscle wasting in this context. To achieve this, we utilised a murine model of AE-COPD with global transgenic deletion of 11β-HSD1 (11βHSD1/KO). Our data show an exacerbation of skeletal muscle wasting during AE-COPD in mice with transgenic deletion of 11β-HSD1 compared to wild type counterparts. This coincides with an elevation of circulating endogenous GC levels and an attenuation of anabolic recovery mechanisms in these 11βHSD1/KO animals following the acute exacerbation. These data offer new insights into the role of endogenous GCs metabolism in muscle atrophy and the efficacy of targeting 11β-HSD1 in the management of muscle wasting in AE-COPD.

3.2 Materials and Methods

3.2.1 Animal Models

To determine the role of 11β-HSD1 in skeletal muscle wasting during an acute exacerbation of COPD, WT and global 11β-HSD1 knock out (11βHSD1/KO) mice were utilised. Global 11βHSD1/KO mice were achieved using Cre-loxP technology, generating a Tri-loxed 11β-HSD1 allele by flanking exon 5 with LoxP sites, as previously described and maintained on a C57BL/6J background [25]. Male WT and 11βHSD1/KO mice (aged 14-20 weeks, n=33) received 2 weekly intra-tracheal instillations (IT) (D0, D7), with 3 U of porcine pancreas elastase (E) (Elastin Products

Company, Missouri, USA) dissolved in 50 µl of sterile $PBS^{-/-}$ to induce emphysema. Presence of emphysema was determined by micro–Computed Tomography (µCT) analysis (D12). WT and 11βHSD1/KO animals were then divided randomly into two sub-groups (n=7-9), receiving a single bolus (IT) of 2µg/g mouse of LPS (Escherichia coli, 055:B5, Sigma, Dorset, UK) dissolved in sterile $PBS^{-/-}$ or $PBS^{-/-}$ alone (v.c.) to evoke a pulmonary inflammatory response (AE) [26, 27]. Animals were sacrificed 48 hours post LPS or PBS IT-instillation and hind limb muscles (gastrocnemius, soleus, tibialis anterior, extensor digitorum longus (EDL) and plantaris) dissected, weighed and snap frozen in liquid nitrogen and stored at -80°C for biochemical analysis. Bloods were collected from abdominal vena cava to obtain plasma. Right lungs lobes were rinsed with hanks balanced salt solution (HBSS) to obtain bronchoalveolar lavage fluid (BALf), left lung lobes were collected and snap frozen for biochemical analysis. All animals were socially housed (n=2-4/cage) in standard conditions (12h/12h dark-light cycle, temp 21 ± 1°C) with ad lib access to standard chow and water. All experiments were carried out at the animal housing facility at Maastricht University. Protocols and procedures involving mice were approved by the Institutional Animal Care Committee of Maastricht University and the Central authority to scientific procedures on animals (CCD) (AVD1070020198766). Group size was determined by power calculations based on previous experience with this model. All analysis derived from animal experiments were analysed blinded.

3.2.2 *µCT imaging and assessment of emphysema and muscle mass*

Animals were anesthetized using a mixture of air and isoflurane (4% induction, 2% maintenance) and scanned using a micro cone beam CT (µCBCT) scanner (XRAD-225Cx, Precision X-Ray, North Branford, USA) at an X-ray tube potential of 50 kVp and an X-ray current of 5.6 mA, giving an image dose of 0.3 Gy. The µCBCT projection data was reconstructed using the Feldkamp back projection algorithm with a voxel dimension of 100 x 100 x 100 µm3 [28]. A mouse-mimicking CT calibration phantom with 12 cylindrical tissue-mimicking inserts of 3.5 mm diameter (SmART Scientific Solutions, Maastricht, the Netherlands) [29] was used to also calibrate the µCBCT scanner in mass density (g/cm3) units. Next, the lung volumes were manually segmented on every reconstructed µCBCT using the SmART-ATP software (version 1.3.6, Precision X-ray Inc.) [30], and the density of every segmented lung voxel was plotted in a mass density histogram. Low attenuation area (LAA) threshold was set from -871 (0.21 g/cm3) to -610 (0.45 g/cm3) Hounsfield units (HU). Muscle volumes were established using the convolutional neural network as previously described [31].

3.2.3 *Assessment of lung and systemic inflammation*

BALf and pellet protein concentrations were measured using BCA Protein Assay Reagent (Fisher Thermo Scientific, Rockford, IL). NF-κB luciferase-reporter cells [32] were grown on Matrigel (Corning, Amsterdam, NL) in in growth medium (GM)

(Low-glucose (1g/L) DMEM containing FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin) for 24 hours and differentiated into myotubes with differentiation media (DM) for 5 days. Myotubes were incubated with condition medium (CM) obtained through diluting BALf or in differentiation medium (DM) (High glucose (4,5g/L) Dulbecco's Modified Eagle's Medium (DMEM) containing 1% heat inactivated FBS, 50 U/ml penicillin, 50 mg/ml streptomycin) for 6 hours at 37 °C. Myotubes were harvested in cell culture lysis buffer (Promega, Madison, USA). Luciferase activity was measured by addition of 100 µL luciferase assay reagent (1.07 mM MgCO3, 2.67 mM MgSO4, 20.0 mM Tricin, 0.10 mM EDTA, 33.3 mM DTT, 530 µM ATP, 270 µM Coenzyme A, 470 µM Luciferin) to 20 µL lysate, and subsequent luminescence detection using a single tube luminometer (Lumat LB 9507, Berthold, Bad Wildbad, Germany), according to the manufacturer's protocol (Promega). The luminescence signal (RLU) was corrected for total protein content in the soluble fraction assessed by a BCA protein assay (Pierce Biotechnology, Rockford, IL).

3.2.4 ELISA Analysis

Serum corticosterone (R&D Systems Parameter™ KGE009, Minneapolis, USA) and IL-6 (R&D Systems Quantikine™ M6000B, Minneapolis, USA) levels were determined using a commercially available ELISA assay in accordance with manufacturer's instructions and optical density was determined at 450 nm using a microplate reader.

3.2.5 RNA isolation and Analysis of Gene Expression

Lung and muscle (gastrocnemius) RNA was extracted by mechanical suspension and lysis of powdered tissue in TRIzol™ reagent (Sigma-Aldrich Chemie B.V, Netherlands). Phase separation and RNA precipitation was performed with the addition of isopropanol (Sigma-Aldrich Chemie B.V, Netherlands) and glycogen (Invitrogen 10814). RNA precipitates were washed in 70% ethanol and reconstituted in RNA storage solution (Invitrogen AM700) and stored at −80°C.

Lung cDNA was generated using the Tetro cDNA Synthesis Kit (GC biotech) according to manufacturer's instructions. Expression of specific genes was assessed by real-time Polymerase chain reaction (PCR) on a LC480 software (Version 2014.1) and relative DNA starting quantities of the samples were derived using LinRegPCR software (Version 2014.0, Ruijter). Expression of genes of interest (Table 1) were normalised using GeNorm software by geometric average of three reference genes (Cyclophilin, RPLP0, and YWHAZ).

Muscle cDNA was generated using the by Multiscribe™ reverse transcription kit (Thermo Fisher Scientific, Loughborough, UK) according to manufacturer's instruction. Expression of specific genes was assessed by real-time PCR using TaqMan® Gene Expression Assays (Thermo Fisher Scientific, Loughborough, UK) on an ABI7500 system (Applied Biosystems,Warrington, UK). Expression of genes of interest (Thermo Fisher Scientific, Loughborough, UK) Fbxo32 (*Atrogin-1, Mm00499523), Trim63 (Murf-1, Mm01185221), Igf-1* (*Mm00439560*)

were normalised using GeNorm software by geometric average of three reference genes (GAPDH, HPRT, and YWHAZ). All data were expressed as arbitrary units (AU) using the calculation; DDCt = DCt[experimental group] - DCt[control group] and reported as fold change = 2DDCt.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Cyclophilin	TTCCTCCTTTCACAGAATTATTCCA	CCGCCAGTGCCATTATGG
RPLP0	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
YWHAZ	TGCTGGTGATGACAAGAAAGGAA	AACACAGAGAAGTTGAGGGCCA
TNF-α	CAGCGCTGAGGTCAATCTGCC	TGCCCGGACTCCGCAA
IL-6	TGAAAATTTCCTCTGGTCTTCTGG	TGGGAGAAGCTTGGGCGTTAA
CXCL1	TCGTCTTTCATATTGTATGGTCAACACG	TGCCCTACCAACTAGACACAAAATGTC
IL-1β	AGCTCTCCACCTCAATGGACAGA	GCCCAAGGCCACAGGTATTTTG

Table 1: Sequence of primers used for RT-qPCR to assess gene expression

3.2.6 Western Blot and Analysis of Protein

Powdered gastrocnemius muscle was lysed in ice-cold IP-lysis buffer containing protease inhibitors (Complete; Roche Nederland, Woerden, Netherlands), using a rotating blade tissue homogenizer (Polytron homogenizer, Kinematica). Total protein concentration of the supernatant was determined with a BCA protein assay kit (Pierce Biotechnology, #23225, Rockford, IL) according to manufacturer's instructions. Proteins were denatured in Laemmli buffer 100°C for 5

min. 10 µg of protein were separated on a CriterionTM XT Precast 4–12% or 12% Bis-Tris gel (Bio-Rad Laboratories, Veenendaal, Netherlands) and transferred onto nitrocellulose transfer membrane (Bio-Rad Laboratories). The membrane was stained with Ponceau S solution (0.2% Ponceau S in 1% acetic acid; Sigma-Aldrich Chemie) to control for protein loading. After blocking in 5% non-fat dried milk, membranes were incubated at 4°C overnight with primary antibodies (Table 2). All antibodies were diluted 1:1000 in Tris Buffered Saline (TBS)-Tween plus 5% Bovine serum albumin (BSA) or 3% skimmed milk. Signal detection used horseradish peroxidase-conjugated secondary antibody (1:10000 in non-fat fried milk) (Vector Laboratories, Burlingame, CA) and visualized with chemiluminescence (Supersignal West Pico or Femto Chemiluminescent Substrate; Pierce). Membranes were imaged (Amersham Imager 600, GE Life Sciences) and quantified using Image Quant software.

Antibody	Cat. Number (Cell Signalling, UK)
p-ULK1 (S757)	#6888
ULK1	#8054
p-FoxO1 (S256)	#9461
FoxO1	#2880
p-Akt (S473)	#9271
Akt	#9272
p-S6 (S235/236)	#4858
S6	#2217
4E-BP1	#9452

Table 2: Antibodies used for western blot

3.2.7 *Assessment of postnatal myonuclear accretion*

Blood was centrifuged and plasma collected. Cre-C2C12 (Cre-IRES-PuroR) cells were differentiated for 4 days, and myotube damaged was induced on day 4 by incubating with HBSS in DM (50%). After 25 hours, LV-floxed-Luc (LV-flox-luc) myoblasts were added cells to Cre-C2C12 myoblasts and further incubated with culture medium (5% serum) for 3 days.

Proliferating Cre-C2C12 myoblasts were cultured as above with the absence of myotube damage on day 4. On day 5, LV-flox-Luc cells were added to C2C12 myoblasts. Following a 6-hour incubation, myotubes were incubated with corticosterone (250nM) (Sigma-Aldrich Chemie B.V, Netherlands, C2505) or dexamethasone (10µM) (Sigma-Aldrich Chemie B.V, Netherlands, D8893) dissolved in DMSO for 3 days.

Luminescence was determined using a luminometer (Berthold Lumat LB9507, Belgium) and corrected for protein content.

3.2.8 *Human myotube culture*

Reagents were obtained from Sigma (Gillingham, UK) unless stated otherwise. Primary myoblasts (CC-2580; Lonza, Slough, UK) were maintained in house in Skeletal Muscle Basal Medium-2 (Lonza; CC-3244 and CC-3246) containing 0.1% human epidermal growth factor, 2% l-glutamine, 10% fetal bovine serum (FBS) and 0.1% gentamicin/amphotericin-B in the absence of GCs. Confluent myotubes

were differentiated in Dulbecco's modified Eagle's medium (DMEM) containing 2% horse serum (HS) for 120 h. Media were replaced every 2–3 days as previously reported [16].

3.2.9 Statistical Analysis

Data are shown as means ± SE. Comparisons were computed using GraphPad Prism [33]. Significance assessment was analysed by unpaired t-test, one way and two-way ANOVA with Tukey post-hoc analysis or Pearson correlation analysis, or non-parametric equivalent tests as appropriate. Statistical significance was defined as p-value < 0.05 (* p < 0.05; ** p < 0.01; *** p < 0.001; no asterisk or NS p > 0.05).

3.3 Results

3.3.1 Confirmation of AE-COPD model in WT and 11βHSD1/KO mice

To investigate the role of 11β-HSD1 in muscle wasting during AE-COPD, WT and 11βHSD1/KO mice were intra-tracheally instilled with elastase, followed by instillation of LPS or vehicle control to evoke a pulmonary inflammatory response in an emphysematous background [27, 34]. To determine emphysema development in both WT and 11βHSD1/KO animals following intra-tracheal instillation of elastase, µCBCT scans were taken (Figure 1A) and lung density histograms were made for each mouse lung (Figure 1B). In line with the left-ward shift suggesting a decrease in the

density of the lung tissue, increased LAA of the lungs on day 12 (D12) compared to any prior elastase treatments on D-1 confirmed the presence of emphysema in WT and 11βHSD1/KO mice (fold change WT = 1.5, $p<0.001$; 11βHSD1/KO = 1.9, $p<0.001$), with no significant differences seen between animal genotypes (Figure 1C).

Subsequently, emphysematous WT and 11βHSD1/KO animals were intra-tracheally instilled with either LPS, to evoke a pulmonary inflammatory response, or PBS. Inflammatory gene expression markers *IL-1RA* (fold change WT = 15.3, p=0.08; 11βHSD1/KO = 8.2, p=0.17; Figure 1D.) and *CXCL2* (fold change WT = 55, p=0.09; 11βHSD1/KO = 41, p=0.24; Figure 1E) in lung tissue of mice treated with LPS were increased compared to WT counterparts, although showed no statistical significance.

In addition, total protein obtained from BALf pellet was increased in response to LPS in WT and 11βHSD1/KO mice (WT = $p<0.05$; 11βHSD1/KO = p=0.051), suggestive of similar inflammatory cell infiltration in the lumen of the lungs of WT and 11βHSD1/KO mice following LPS (Figure 1F).

To assess the systemic impact of AE-COPD, body weights were assessed 48 hours post-installation (Figure 1G). Both WT and 11βHSD1/KO animals showed significant reduction in body weights following LPS treatment compared to PBS controls (-11%, $p<0.05$ and -15%, $p<0.05$ respectively), with no significant differences seen between LPS-treated WT and 11βHSD1/KO animals.

Combined, these data demonstrate that the lung inflammatory response and body weight loss as a systemic consequence of AE-COPD are preserved in emphysematous 11βHSD1/KO mice.

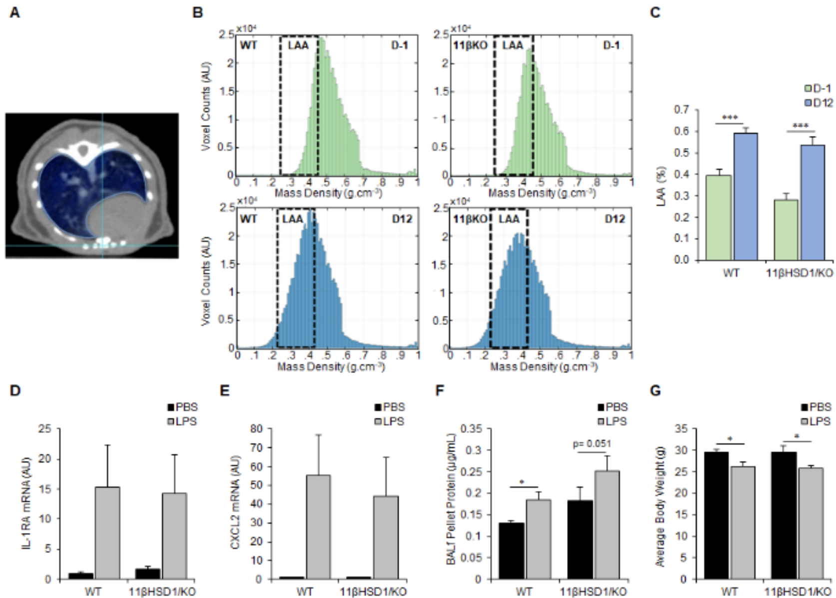

Figure 1: WT and 11β-HSD1/KO mice intra-tracheally instilled with elastase and LPS developed emphysema and pulmonary inflammation respectively. µCT scans were obtained one day before (D-1) and 12 days following (D12) intra-tracheal instillation of elastase in WT and 11βHSD1/KO mice (A) and were analysed for LAA, with a LAA threshold of -871 (0.21 g/cm^3) to -610 (0.45 g/cm^3) Hounsfield units (HU) (B-C). Mice then received a single bolus of PBS or LPS (n = 7-9 per group) and lungs and BALf collected after 48 h for mRNA and protein analysis. Gene expression (AU) of Lung IL-1RA (D) and CXCL2 (E) were determined and expressed as fold change compared to control. Total protein concentrations of BALf pellet (F) were determined and expressed as fold change compared to control. Body weights were taken before sacrifice (G) expressed as mean ± standard error. Statistical significance was determined using two-way analysis with Tukey post hoc analysis and unpaired t-test. * p<0.05, *** p<0.001. WT, wild type; 11βHSD1/KO, 11βHSD1 global genetic deletion; LAA, low attenuation area; AU, arbitrary units; BALf, bronchoalveolar lavage fluid.

3.3.2 *Muscle wasting is more pronounced in LPS-treated emphysematous 11βHSD1/KO mice.*

To investigate the involvement of 11β-HSD1 in the impact of lung inflammation on muscle wasting in this model [26, 27], hind limb muscle mass was calculated using non-invasive µCBCT scans of hind limb muscles 48 hours post-LPS instillation (Fig 2A). CT-derived muscle mass was reduced in both WT (-4%, $p > 0.05$; Figure 2B) and 11βHSD1/KO animals (-14%, $p < 0.05$; Figure 2B) treated with LPS. Accordingly, wet weight of the M. gastrocnemius was significantly reduced in LPS-treated compared to PBS control animals of both genotypes (-7%, $p < 0.005$ and -13%, $p < 0.0001$ respectively).

In order to determine the effects of AE-COPD on muscle wasting in WT and 11βHSD1/KO animals we examined *Atrogin-1* and *MuRF-1* expression levels as these E3 ligases are involved in muscle proteolytic responses [35]. LPS treatment significantly induced *Atrogin-1* mRNA levels in the gastrocnemius muscle after 48 hours in both WT and 11βHSD1/KO mice (fold change WT = 2.5, $p<0.05$; 11βHSD1/KO = 5.1, $p<0.005$; Figure 2D). *Atrogin-1* mRNA levels were increased in LPS treated WT and 11βHSD1/KO animals, which was slightly pronounced, though not significantly, in 11βHSD1/KO mice compared to WT controls (fold change = 1.9, $p=0.11$). *MuRF-1* levels were increased in 11βHSD1/KO mice in response to LPS and were significantly greater than WT controls (fold change 11βHSD1/KO = 3.2, $p=0.07$; Figure 2E).

Combined, these data show more pronounced muscle wasting in 11βHSD1/KO animals following AE-COPD.

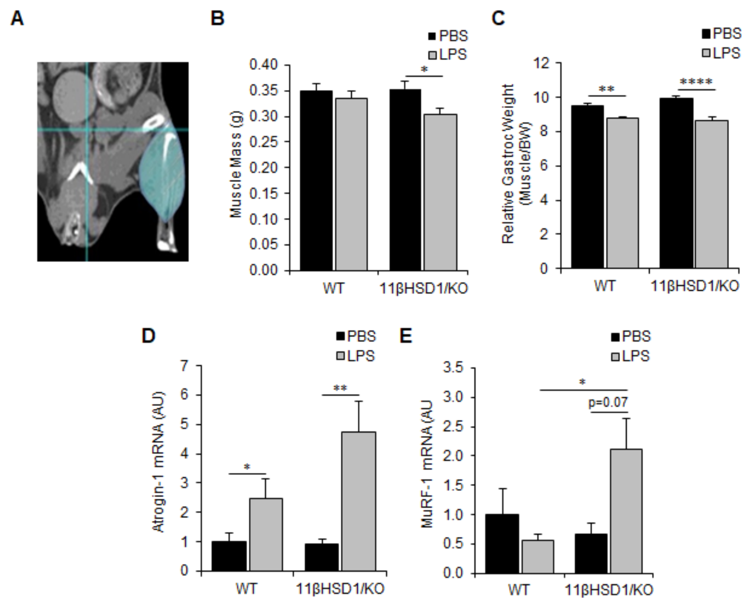

Figure 2: LPS-treated emphysematous 11βHSD1/KO mice show exacerbated muscle wasting compared to WT controls. 48 hours after PBS or LPS treatment, µCT scans were taken (**A**) and muscle mass of total hind limb (**B**) was determined. Gastrocnemius wet muscle weights were measured and expressed relative to start body weight (**C**). Gene expression (AU) of homogenised gastrocnemius Atrogin-1 (**D**) and MuRF-1 (**E**) levels were determined and expressed as fold change compared to control. Values are expressed as mean ± standard error. Statistical significance was determined using two-way analysis with Tukey post hoc analysis and unpaired t-test. * $p<0.05$, ** $p<0.005$, **** $p<0.0001$. WT, wild type; 11βHSD1/KO, 11βHSD1 global genetic deletion; Gastroc, gastrocnemius; BW, body weight.

3.3.3 Suppression of catabolic signalling is reduced in the muscle of 11βHSD1/KO compared to WT animals in response to LPS.

For further insight into catabolic processes in the gastrocnemius muscle, we examined protein levels and phosphorylation status of markers of the autophagy lysosomal pathway using western blot analysis (Figure 3A-G). ULK1 (Ser757) phosphorylation inhibits autophagosome formation [36], and was increased in both WT and 11βHSD1/KO mice in response to LPS (fold change WT = 1.4, $p<0.05$; 11βHSD1/KO = 1.3, p=NS; Figure 3A). In comparison, total levels of ULK1 were slightly elevated with LPS treatment in WT than 11βHSD1/KO animals (fold change WT = 1.5, p=NS; 11βHSD1/KO = 1.1, p=NS; Figure 3B). These increases were not reflected in p-ULK1/Total ULK1 ratios (Figure 3C), suggesting attenuation of autophagic signalling. Transcriptional activity of FoxO1 is inactivated through phosphorylation on Ser256 by Akt [37]. Treatment with LPS increased phosphorylation of FoxO1 (Ser256) (fold change WT = 3.9 $p<0.001$; 11βHSD1/KO = 3.5 $p<0.001$; Figure 3D) and Total FoxO1 (fold change WT = 1.9 $p<0.005$; 11βHSD1/KO = 2 $p<0.001$; Figure 3E) protein levels across both groups. Despite this, there was a clear increase in FoxO1 p/total ratio in both WT and 11βHSD1/KO mice with LPS treatment (fold change WT = 1.9 $p<0.005$; 11βHSD1/KO = 1.7 p=NS; Figure 3F).

These data show suppression of protein degradation signalling in muscle of LPS-treated mice, which appears slightly attenuated in 11βHSD1/KO compared to WT animals.

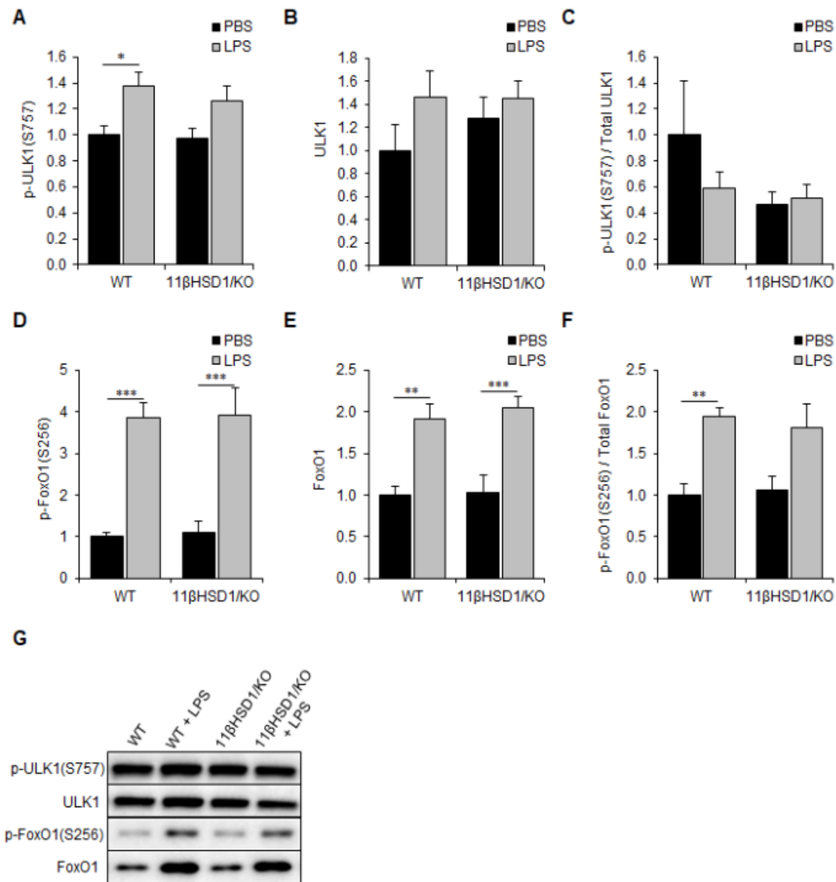

Figure 3: Catabolic signalling in the muscle of WT and 11βHSD1/KO animals in response to LPS. 48 hours after PBS or LPS treatment, gastrocnemius muscle was collected for mRNA and protein analysis. Gastrocnemius protein levels of phosphorylated ULK1(S575) (**A**) and FoxO1(S256) (**D**), and total ULK1 (**B**) and FoxO1 (**E**) were assessed, normalised to Ponceau staining and expressed as fold change compared to control with representative western blot images (**G**). Ratios of phosphorylated and total ULK1 (**F**) and FoxO1 (**I**) were assessed and shown as fold change compared to control. Statistical significance was determined using two-way analysis with Tukey post hoc analysis and unpaired t-test. * $p<0.05$, ** $p<0.005$, *** $p<0.001$. WT, wild type; 11βHSD1/KO, 11βHSD1 global genetic deletion; AU, arbitrary units.

3.3.4 *Anabolic signalling is activated in the muscle of WT compared to 11βHSD1/KO emphysematous mice following LPS.*

We next also determined the impact of AE-COPD on anabolic signalling in the gastrocnemius muscle of WT and 11βHSD1/KO animals. FoxO1 was investigated as an upstream regulator of protein synthesis (Figure 4A-C, 4G) [37]. In LPS-treated WT mice, a significant increase in phosphorylation of Akt was observed (fold change = 1.5, $p<0.005$; Figure 4A), which was not apparent in 11βHSD1/KO animals. Although this differential increase was also observed for p/total Akt ratio (Figure 4C), it was not significant due to small changes in total Akt levels (Figure 4B). A similar pattern was observed in total Akt levels, although not significant (WT $p=0.39$; 11βHSD1/KO $p>0.99$; Figure 4B). Furthermore, a trend of increased ratio levels of p/total Akt was observed in both WT and 11βHSD1/KO mice (fold change WT = 1.3, $p=0.008$; 11βHSD1/KO = 1.1, $p=0.89$; Figure 4C). In addition, phosphorylation status of S6 (Ser235/236), which determines mRNA translation in protein synthesis, was examined (Figure 4D-F). Both WT and 11βHSD1/KO animals treated with LPS showed increases in p-S6, with the former having a significant increase (fold change WT = 1.7, $p<0.005$; 11βHSD1/KO = 1.7, p=NS; Figure 4D). This pattern was also apparent for p/total S6 ratios (fold change WT = 1.9, $p<0.05$; 11βHSD1/KO = 1.8, $p=0.18$; Figure 4F) and hyper-phosphorylation of 4E-BP1 (fold change WT = 2.9, $p<0.01$; 11βHSD1/KO = 4, $p<0.05$; Figure 4H).

IGF-1 and subsequent Akt phosphorylation within muscle are potent drivers of anabolic protein synthesis [38], therefore we explored IGF-1 gene expression in the gastrocnemius muscle (Figure 4I). Interestingly, higher levels of IGF-1 mRNA were observed in WT animals given LPS (fold change = 1.9, $p=0.20$) compared to no effect of LPS on IGF-1 in 11βHSD1/KO mice. Taken together, these data may suggest that 11βHSD1/KO mice treated with LPS have a delayed reactivation of protein synthesis signalling and reduced initiation of a recovery response in skeletal muscle compared to WT counterparts.

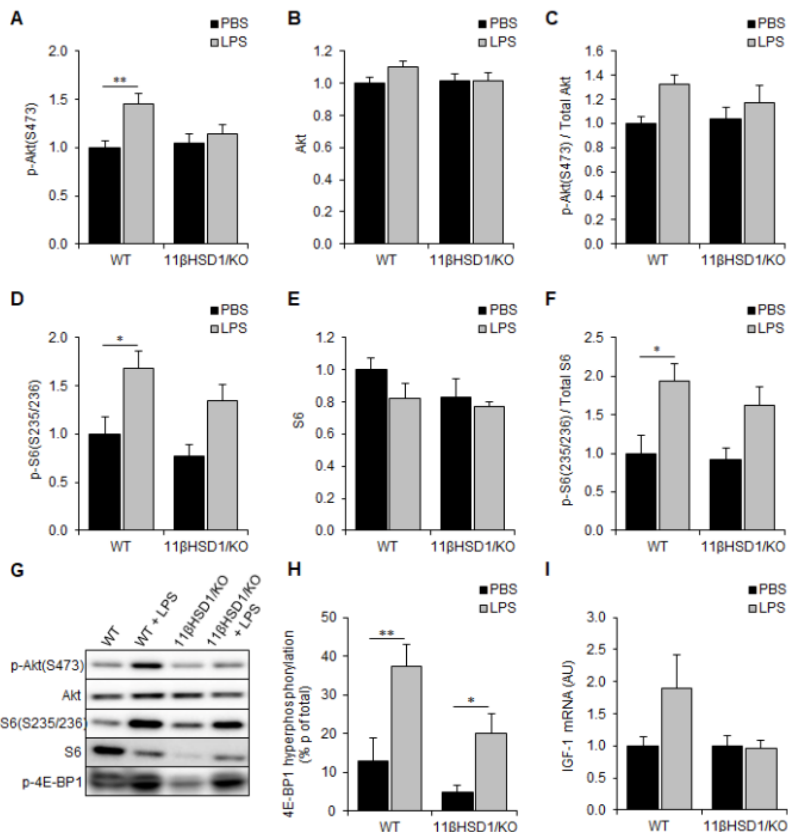

Figure 4: Anabolic signalling in the muscle of WT and 11βHSD1/KO animals in response to LPS. 48 hours after PBS or LPS treatment, gastrocnemius muscle was collected for mRNA and protein analysis. Gastrocnemius protein levels of phosphorylated Akt(S475) (**A**) and S6(s235/236) (**D**), and total Akt (**B**) and S6 (**E**) were assessed, normalised to Ponceau staining and expressed as fold change compared to control with representative western blot images (**G**). Ratios of phosphorylated and total Akt (**C**) and S6 (**F**) were assessed and shown as fold change compared to control Hyper-phosphorylation of 4E-BP1 were assessed (**H**). Gene expression (AU) of IGF-1 (**I**) from homogenised gastrocnemius determined by RT-qPCR normalised to GeNORM and expressed as fold change compared to control. Statistical significance was determined using two-way analysis with Tukey post hoc analysis and unpaired t-test. * p<0.05, ** p<0.005. WT, wild type; 11βHSD1/KO, 11βHSD1 global genetic deletion; AU, arbitrary units.

3.3.5 Serum corticosterone levels are increased in LPS treated 11βHSD1/KO animals and suppress in vitro myonuclear accretion.

To investigate whether altered muscle recovery responses in WT and 11βHSD1/KO mice reflect actions of circulating mediators, we examined the impact of the serum of these animals in a C2C12 model of myonuclear accretion. Mouse plasma was added to damaged C2C12-Cre myotubes co-cultured with LV-flox-Luc C2C12 myoblasts and postnatal myonuclear accretion, a process essential for skeletal muscle growth and repair, was assessed (Figure 5A). Both WT and in 11βHSD1/KO mice treated with LPS showed a slight increase in myonuclear accretion (Figure 5B), trending towards significance in WT animals. Instead, serum corticosterone levels were increased but only in LPS-treated 11βHSD1/KO mice compared to WT controls (fold change = 1.4, $p<0.05$; Figure 5C) and direct comparison between LPS treated WT and 11βHSD1/KO mice showed serum corticosterone levels significantly higher in 11βHSD1/KO than WT animals (fold change = 1.5, $p<0.005$). Importantly, increased gastrocnemius mRNA levels of *Gilz*, a glucocorticoid responsive gene, were only observed in LPS-treated 11βHSD1/KO mice (fold change WT = 1.2 $p=0.57$; 11βHSD1/KO = 1.8 $p<0.05$; Figure 5D), indicative of CORT-induced signalling in affected skeletal muscle. Human primary cultures treated with corticosterone showed significant increases in Gilz and FoxO1 mRNA expression (Figure 5E-F). We then examined the impact of elevated corticosterone levels in a murine primary myotube and C2C12 culture. *In vitro* investigation of the

effects of GCs on postnatal myonuclear accretion showed significant inhibition by both corticosterone and synthetic GC dexamethasone (fold change CORT = 0.5 $p<0.05$; DEX = 0.3 $p<0.005$; Figure 5G).

These data demonstrate that 11βHSD1/KO animals have a greater GC response to LPS than WT counterparts *in vivo*. In addition, GCs can impair myonuclear accretion in vitro, therefore may impair muscle recovery following damage.

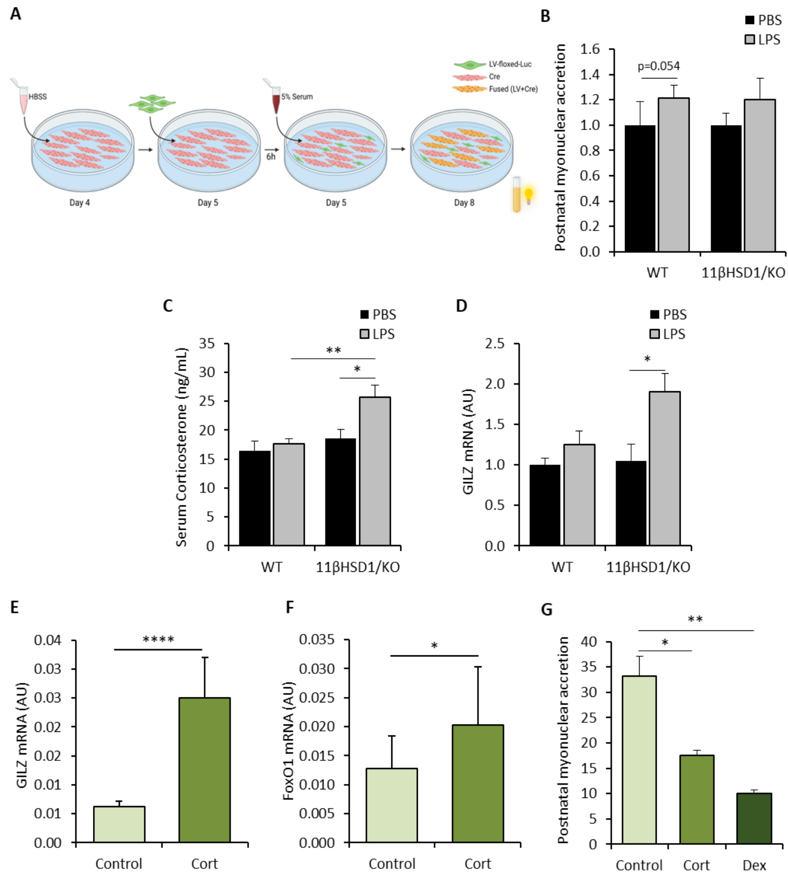

Figure 5: Glucocorticoid levels in serum and muscle LPS treated WT and 11βHSD1/KO animals and in vitro analysis of myonuclear accretion when treated with glucocorticoids. *In vitro* postnatal myonuclear accretion of damaged C2C12 myotubes incubated with mouse serum was determined by luciferase activity (A-B) (Created with BioRender). Serum corticosterone levels determined by ELISA (C). Gene expression (AU) of GILZ from homogenised gastrocnemius expressed as fold change compared to control (D). Gene expression (AU) of GILZ (E) and FoxO1 (F) of *ex vivo* human primary myotubes treated with cortisol (100nM) (n=3). Effects of corticosterone (250nM) or dexamethasone (10µM) (72 h) on postnatal myonuclear accretion *in vitro* (n = 3) (G), relative luciferase activity (RLU/protein). Statistical significance was determined using two-way analysis with Tukey post hoc analysis and unpaired t-test. * p<0.05, ** p<0.005. WT, wild type; 11βHSD1/KO, 11βHSD1 global genetic deletion; AU, arbitrary units; Cort, corticosterone; Dex, dexamethasone.

3.4 Discussion

Both enhanced systemic inflammation and elevated glucocorticoid responses have been implicated as important drivers of muscle wasting [39-43] in acute exacerbations of COPD (AE-COPD) and pulmonary inflammation-induced muscle loss. 11beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) is a pre-receptor regulator and gatekeeper of GC action, and its role in this context remains poorly defined. The aim of this study was to determine the contribution of 11β-HSD1 in muscle atrophy associated with acute exacerbations of COPD and test the hypothesis that its transgenic deletion in a mouse model of AE-COPD can abrogate GC-induced muscle wasting in this disease model setting.

In this study, we deployed a murine model of AE-COPD driven by repeated elastase instillation and LPS induction of pulmonary inflammation, characterised by emphysema, pulmonary and systemic inflammation and muscle atrophy [27]. The majority of AE-COPD are induced by bacterial or viral infections (59%) [44], therefore whilst our model utilises the bacterial component LPS to induce pulmonary and systemic inflammation, further studies investigating other potential triggers of AE, such as viral pathogens, cigarette smoke and pollution are required to improve translatability more widely to AE-COPD patients. We observed increased muscle atrophy in animals with global deletion of 11β-HSD1 following AE-COPD relative to their wild-type counterparts, characterised by increased measures of Ubiquitin 26S-proteasome (UPS) mediated degradation and suppressed markers of muscle

anabolism and recovery. These findings were contrary to the original hypothesis based in murine models of corticosteroid excess, where we postulated that global deletion of 11β-HSD1 would protect from the anti-anabolic/catabolic actions of corticosteroids in muscle [45, 46]. Instead, they more closely reflected observations of exacerbated muscle wasting previously reported in murine models of chronic inflammation with global deletion of 11β-HSD1, where the loss of local glucocorticoid reactivation fuelled increased catabolic wasting [16]. The most evident cause of the discrepancy between these studies may relate to the dosing of corticosteroid exposure, where 11β-HSD1 deletion protected from muscle wasting at higher therapeutic doses and exacerbated wasting when endogenous corticosteroid levels were examined. In this study, the increased muscle wasting observed in animals with transgenic deletion of 11β-HSD1 correlated with a marked increase in circulating endogenous corticosteroids and muscle GR signalling in response to AE-COPD.

Endogenous circulating GCs are significantly upregulated through the hypothalamic-pituitary-adrenal (HPA) axis, in response to various acute inflammatory stressors, including sepsis and the acute exacerbation of COPD [47, 48]. Whilst elevated levels of endogenous circulating GCs support the suppression of systemic inflammation and improve survival outcomes [49], the increased activation of GC signalling in skeletal muscle drives proteolysis and reduced anabolic signalling. Previous studies have implicated the importance of GC signalling in mediating muscle

atrophy in murine models of sepsis and inflammation-associated muscle wasting [50]. Here, the targeted deletion of GR in mouse muscle showed prevention of muscle wasting in models of LPS-induced sepsis and in cancer-associated muscle wasting [50]. Similarly, atrogene expression in rat muscle was blunted in response to LPS with inhibition of GR using RU-486 [51], further emphasising the importance of GC signalling in skeletal muscle with inflammatory-associated muscle wasting.

The enzyme 11β-HSD1 mediates cellular GC action through its conversion of inactive GCs to their active counterparts [52]. In prior studies, its transgenic deletion in models of corticosterone excess resulted in a marked protection from GC induced muscle wasting (including in models of inflammatory polyarthritis), preventing the activation of GC-induced catabolic pathways including the transcription factor FoxO1 and muscle-specific E3 ligases Atrogin-1 and MuRF-1 [45, 46]. In contrast, its transgenic deletion in chronic models of TNF-α driven inflammatory polyarthritis in the absence of exogenous therapeutic GCs exacerbates inflammation-induced muscle wasting [16]. These studies reveal the duality of the roles of 11β-HSD1 in muscle in inflammatory disease, where it mediates the muscle protective anti-inflammatory properties of endogenous GCs, as well as mediating direct GC induced muscle wasting in response to exogenous therapeutic GCs [16].

Whilst activation of the NF-κB pathway within muscle has been shown to be an important component of muscle wasting in following pulmonary inflammation [13], transgenic deletion of 11β-HSD1 did not enhance inflammatory signalling

within muscle (Supplementary Figure S1) [13]. These results would indicate that 11β-HSD1 appears to play a limited role in suppressing local muscle inflammation, and inflammatory muscle wasting in this model of AE-COPD. Systemic markers of inflammation showed a similar trend across both WT and 11BKO animals, with levels of IL-6 in response to LPS showing a comparable induction in both groups, suggesting that the severity of AE inflammation did not appear to mediate the differences in muscle wasting observed between WT and 11βHSD1/KO animals in this model (Supplementary Figure S2).

However, whilst measures of inflammation were comparable between WT and 11βHSD1/KO animals, circulating levels of endogenous GCs showed a significant divergence with corticosterone being significantly elevated in 11βHSD1/KO animals following AE-COPD. This was accompanied by a significant increase in measures of GC signalling in muscle, with a marked increase in the response gene *Gilz* in 11βHSD1/KO animals [53]. Together these results suggest that the muscles during AE-COPD, from 11βHSD1/KO animals are experiencing increased catabolic and anti-anabolic GC signalling as a result of elevated circulating GC exposure. Previously, elevated activation of the HPA axis and GC signalling within muscle have been shown to contribute to muscle atrophy in LPS-induced inflammation [8]. This increased atrophy and GC response indicated that the HPA axis plays a pivotal role in driving inflammatory muscle catabolism. Pro-inflammatory mediators such as IL-6 and TNF-α are potent drivers of HPA axis activation and corticosteroid release [54]. This

process of HPA axis upregulation by pro-inflammatory mediators is in turn negatively regulated by circulating GCs in a negative feedback loop that facilitates resolution of circulating GCs levels. 11β-HSD1 has been shown to be highly expressed, and dynamically regulated within the hypothalamus, with its local amplification of GCs playing a role facilitating negative feedback of circulating corticosteroids in the HPA axis [55]. This loss of 11β-HSD1 within the hypothalamus in 11βHSD1/KO animals may be one factor resulting in the elevated levels of circulating GCs and muscle wasting during AE-COPD in 11βHSD1/KO animals.

GCs induce muscle atrophy through several pathways, driving anti-anabolic [56, 57], and catabolic signalling primarily through activation of the UPS [35]. Upon exposure to GCs, transcription factor FoxO1 increases in expression and activity [58], in turn activating atrogenes such as Atrogin-1 and MuRF-1 [59]. Increased FoxO1 transcript levels in primary human myotubes in response to cortisol confirm these effects of GCs, and the increases in FoxO1 protein levels in WT and 11βHSD1/KO muscle reflect preceding transcriptional GR actions induced by GCs. FoxO1 activity is subject to regulation of its nuclear export following phosphorylation by Akt [37], and the kinetics of this inhibitory phosphorylation inversely correlate with muscle mass loss in response to pulmonary inflammation [60]. We propose that increased circulating GCs may drive reduced inhibitory FoxO1 phosphorylation in AE-COPD in LPS-11βHSD1/KO animals contributing to the aggravated muscle loss we observe.

In line with previous observations in the related model of pulmonary inflammation, protein synthesis signalling through the IGF-1/Akt/mTOR pathway is reactivated 48h following LPS in muscle of WT animals, which signified initiation of muscle mass recovery in that study [60]. Although mTOR activity was not directly measured in this study, increased phosphorylation of its indirect and direct downstream targets, i.e., S6, 4E-BP1 and ULK1 suggests activation of mTOR signalling in WT muscle. As the phosphorylation of Akt and the downstream mTOR targets is consistently lower or absent, this suggests attenuated protein synthesis signalling in muscle of 11βHSD1/KO compared to WT animals following AE-COPD. Such anti-anabolic effects of GCs on muscle by antagonism of the IGF-1/Akt/mTOR pathway has been well established [56, 57]. *In vivo* treatment of methylprednisolone, a synthetic GC, in male rats showed significant reductions in IGF-1 mRNA expression in the gastrocnemius [61]. It has been previously demonstrated that Akt signalling is impaired by non-genomic actions of GR by GCs, which block IRS-1-PI3K interactions, resulting in reduced Akt phosphorylation and suppression of downstream mTOR signalling [62]. While phosphorylation of S6 kinase and 4E-BP1 are instrumental for initiating mRNA translation, GC treatment of myoblasts significantly reduces S6 phosphorylation, illustrating the direct inhibitory actions of GCs on protein synthesis [56]. Taken together, we speculate attenuated anabolic signalling in LPS-11βHSD1/KO mice compared to WT counterparts reflects the impact of residual elevations in circulating GCs and contributes to reduced muscle mass. However,

further measures of anabolic protein metabolism, such as puromycin incorporation into muscle are required to effectively validate these observations [63]. Unfortunately, these went beyond the scope of the current study.

Another process involved in muscle mass maintenance and recovery is post-natal myogenesis, in which satellite cells differentiate and fuse with existing fibers [64]. Myogenesis has been shown to be inhibited by systemic cues, including inflammatory cytokines and GCs [65], resulting in reduced satellite cell proliferation and differentiation [66], and myonuclear accretion [67]. We modelled postnatal myogenesis driven muscle recovery in vitro to assess the presence of circulating mediators in serum that impact on this process. Myonuclear accretion in response to a standardized atrophic stimulus has a trend towards attenuation in presence of serum from LPS-11βHSD1/KO mice compared to WT counterparts. While no difference in inflammatory cytokine IL-6 levels are present (Supplementary Figure S2), the significant increases in corticosterone serum levels suggest GCs directly interfere with myonuclear accretion. In line with this notion, treatment with corticosterone or dexamethasone is sufficient to inhibit myonuclear accretion. Although not assessed in a postnatal myogenesis set-up as done here, recent studies have shown inhibition of myogenesis in C2C12 cells following dexamethasone and cortisone treatment, in line with our findings [68]. Combined, the elevated circulating GCs levels present in LPS-11βHSD1/KO mice may be responsible for

suppressed protein synthesis signalling and impaired myonuclear accretion, contributing to exaggerated muscle wasting.

Global deletion of 11β-HSD1 allows for translational extrapolation to the use of therapeutic 11β-HSD1 inhibitors. However, our study indicated that this approach does not prevent muscle wasting in the backdrop of AE-COPD. However, a targeted, muscle-specific 11β-HSD1 KO approach would help disentangle deleterious and positive actions of both local and systemic actions of 11β-HSD1.

This study highlights the important role of 11β-HSD1 in mediating muscle wasting during an acute exacerbation of COPD. We show that with the transgenic deletion of 11β-HSD1 during AE-COPD, there is a compensatory increase in circulating corticosterone during the resolution phase of pulmonary and systemic inflammation, which correlates with a sustained activation of UPS-mediated proteolysis and reduced muscle anabolic signalling. Therefore, the use of therapeutic 11β-HSD1 inhibitors during an acute exacerbation of COPD may not be appropriate in this setting.

Author Contributions

Animal experiments and analysis of in vivo and ex vivo data were performed by JMW with support from WvdW, RCJL and SL. In vitro experiments were performed and analysed by JMW, KW, MK. µCT experiments were analysed by JMW and BVdH with support from FV and RL. RCJL, RSH and JW, designed and supervised the project with support from AMWJS and GGL. All authors discussed the results and contributed to the final manuscript. All authors read and approved the final manuscript.

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Conflict Of Interest

The authors reported no conflict of interests for the work described here.

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Supplementary Data

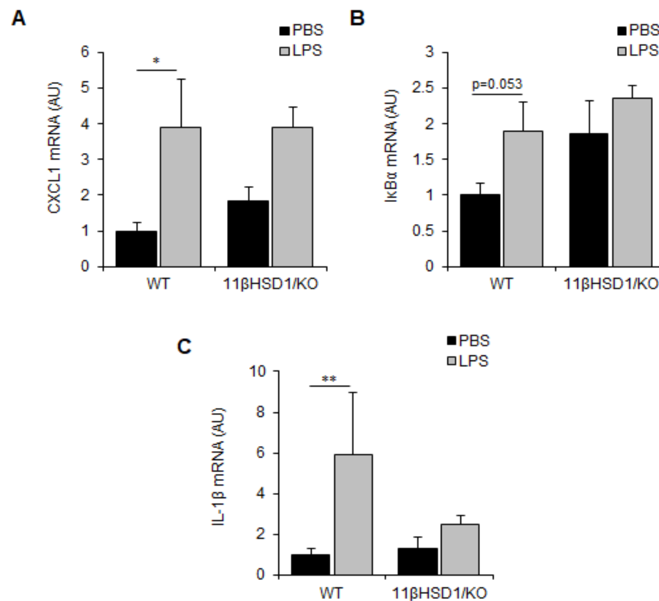

Figure S1: Inflammatory responsive gene expression in gastrocnemius muscle. Gene expression (AU) of CXCL1 (**A**), IκBα (**B**) and IL-1β (**C**) from homogenised gastrocnemius expressed as fold change compared to control. Statistical significance was determined using two-way analysis with Tukey post hoc analysis and unpaired t-test. * p<0.05. WT, wild type; 11βHSD1/KO, 11βHSD1 global genetic deletion; AU, arbitrary units.

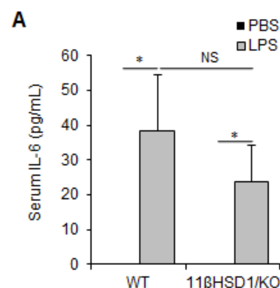

Figure S2: Mouse serum IL-6 levels. Serum IL-6 levels determined by ELISA (**A**). Statistical significance was determined using two-way analysis with Tukey post hoc analysis and unpaired t-test. * p<0.05, NS; Not significant. WT, wild type; 11βHSD1/KO, 11βHSD1 global genetic deletion.

Chapter 4 - Exploring the Interface between Inflammatory and Therapeutic Glucocorticoid Induced Bone and Muscle Loss

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Abstract

Due to their potent immunomodulatory anti-inflammatory properties, synthetic glucocorticoids (GCs) are widely utilized in the treatment of chronic inflammatory disease. In this review, we examine our current understanding of how chronic inflammation and commonly used therapeutic GCs interact to regulate bone and muscle metabolism. Whilst both inflammation and therapeutic GCs directly promote systemic osteoporosis and muscle wasting, the mechanisms whereby they achieve this are distinct. Importantly, their interactions in vivo are greatly complicated secondary to the directly opposing actions of GCs on a wide array of pro-inflammatory signalling pathways that underpin catabolic and anti-anabolic metabolism. Several clinical studies have attempted to address the net effects of therapeutic glucocorticoids on inflammatory bone loss and muscle wasting using a range of approaches. These have yielded a wide array of results further complicated by the nature of inflammatory disease, underlying the disease management and regimen of GC therapy. Here, we report the latest findings related to these pathway interactions and explore the latest insights from murine models of disease aimed at modelling these processes and delineating the contribution of pre-receptor steroid metabolism. Understanding these processes remains paramount in the effective management of patients with chronic inflammatory disease.

Keywords: glucocorticoid, muscle wasting, osteoporosis

4.1 Glucocorticoids And Therapeutic Glucocorticoid Excess

Synthetic glucocorticoids (GCs), such as dexamethasone, prednisolone and hydrocortisone, are widely utilized in the treatment of chronic inflammatory diseases such as chronic obstructive pulmonary disease (COPD), inflammatory bowel disease (IBD) and rheumatoid arthritis (RA), with approximately 1% of the adult population in the U.K. and U.S. receiving this class of drugs. Their mechanisms of action are diverse, with GCs suppressing a range of pro-inflammatory pathways including p38-mitogen activated protein kinases (p38-MAPK), nuclear factor kappa-light-chain-enhancer (NF-κB) and activator protein (AP-1), in addition to inducing pro-resolving factors such as glucocorticoid induced leucine zipper (GILZ) and annexin-1 [1-3]. These significantly reduce leukocyte infiltration at sites of inflammation, suppress the production of pro-inflammatory cytokines and chemokines and support resolution of inflammation and tissue remodelling [4, 5]. Despite the potent immune-modulatory anti-inflammatory actions of therapeutic GCs, their clinical application is limited due to severe systemic side effects. These occur in up to 70% of patients and can include muscle wasting and GC induced osteoporosis (GIO) [6-10]. The actions of GCs on bone and muscle metabolism are well established, but themselves complicated in the backdrop of chronic inflammation by separate inflammatory driven muscle wasting and bone loss. The inflammatory pathways that mediate bone and muscle loss in chronic inflammation are in turn suppressed by the anti-inflammatory actions of GCs, further complicating

the prediction of their outcome on the musculoskeletal system. Understanding the complex interactions between GC and inflammatory regulation of bone and muscle metabolism remains paramount in the effective management of patients with chronic inflammatory disease. In this review, we explore how inflammatory drivers and therapeutic GCs interact to regulate bone and muscle metabolism and consider the role of local steroid metabolism in shaping these processes.

4.2 Glucocorticoid signalling and regulation of inflammation

Lipophilic GCs readily diffuse across cell membranes, signalling through the cytoplasmic GC receptor (GR) superfamily, encoded by the NR3C1 gene. Classically, GC signalling and GR transactivation occur through ligand binding of the GRα homodimer. In its unbound state, GRα forms a multi-protein complex with chaperone proteins such as heat shock proteins (HSPs), HSPp-70, HSP90 and FK506 binding protein 52 that block their nuclear localization signal (NLS) and prevent translocation to the nucleus from the cytoplasm [11]. Upon GC binding, the GRα undergoes a conformational change, allowing dissociation of chaperone proteins. Homo-dimerization and exposure of the NLS are required for nuclear translocation of the ligand bound GR, where it can influence gene expression [12] (Figure 1). This is an oversimplified view of GC signalling, as several studies utilizing the GR^{dim} mouse (possessing a mutation preventing GR dimerization) reveal that the anti-inflammatory properties of therapeutic GCs are mediated by both homo-dimeric

GRα complexes and monomeric GRα to facilitate transactivation or transrepression of pro-inflammatory genes [5, 13-16]. Whilst the mechanisms that underpin GR signalling have been reviewed extensively elsewhere, several key pathways are prominent in mediating the anti-inflammatory actions of GCs [17]. These include the direct GRα homodimer transactivation of anti-inflammatory genes such as secretory leukocyte protease inhibitor (SLPI), MAKP-1, GILZ and tristetraprolin (TTP), which suppress the NF-kB and p38-MAPK inflammatory pathways, in addition to the inhibition of pro-inflammatory transcription factors via their tethering to the GC bound GR [18-21]. In particular, GCs act via the GR to suppress the NF-κB and p38-MAPK inflammatory pathways and AP-1 pro-inflammatory pathways, which regulate the transcription of various genes relating to inflammation such as tumour necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1β) and -6 [22]. Many of these inflammatory pathways considered are direct contributors to the process of inflammatory bone and muscle wasting and are themselves opposed by the actions of therapeutic GCs. This review will now consider how inflammation and GCs influence bone and muscle metabolism, both in isolation and in concert with one another.

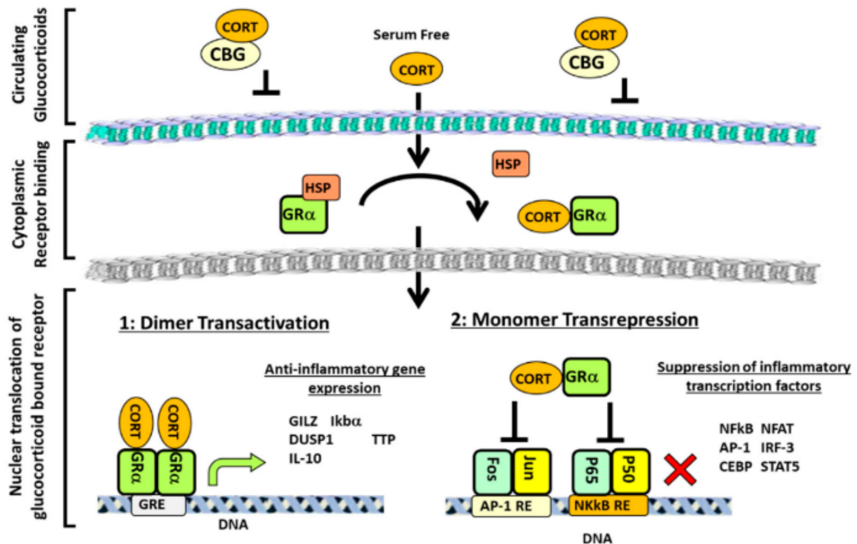

Figure 1. Overview of glucocorticoid (GC) signalling pathways. The majority of glucocorticoids (GCs) in the circulation are bound by corticosteroid-binding globulin (CBG), which prevents diffusion across the membrane. However, free GCs can readily enter the cell, where they bind to the GR in the cytoplasm. This induces a conformational change in the glucocorticoid receptor (GR), which causes the dissociation of chaperone molecules, such as heat shock proteins (HSPs), to expose the nuclear localisation signal (NLS) and allow translocation of the GC/GR complex to the nucleus. Here, the GR can either dimerise to transactivate anti-inflammatory genes or signal as a monomer to inhibit pro-inflammatory transcription factors. Cortisol (CORT), nuclear factor of activated T-cells (NFAT), CCAAT-enhancer-binding proteins (or C/EBPs), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), p38 mitogen-activated protein kinases (p-38-MAPK), glucocorticoid induced leucine zipper (GILZ), secretory leukocyte protease inhibitor (SLPI), tristetraprolin (TTP), mitogen-activated protein kinase-1 (MKP-1), activator protein 1 (AP-1), signal transducer and activator of transcription 5 (STAT5), and response element (RE).

4.3 Bone metabolism

Bone metabolism is a tightly regulated process that ensures homeostasis between bone resorption and bone formation. This process maintains a balance in calcium and phosphate mineral homeostasis, as well as allowing constant healthy remodelling to compensate for external loading stress and damage and requires the close interaction between osteocytes, bone lining cells, bone forming osteoblasts and bone resorbing osteoclasts [23]. Here, in quiescent bone, osteocytes produce factors such as transforming growth factor-β (TGF-β), sclerostin and dickkopf WNT signaling pathway inhibitor 1 (DKK-1), which inhibits osteoclast and osteoblast maturation and differentiation [24]. Signals such as bone matrix damage or immobilization result in osteocyte apoptosis, leading to a removal of the inhibitory signals and increases in factors that promote osteoclastogenesis, such as macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-β ligand (RANKL) [25-27]. Together, these promote osteoclast differentiation from hematopoietic precursors and increase receptor activator of nuclear factor kappa-β (RANK) pathway activation, driving multinuclear polykaryon formation and the formation of mature osteoclasts that express osteoclast specific genes including tartrate-resistant acid phosphatase (TRAP) and cathepsin K [28-30]. Mature osteoclasts form tight integrin junctions on mineralized bone matrix, forming an acidified resorption compartment that facilitates the degradation of the inorganic hydroxyapatite component of the bone [31, 32]. The organic component of bone can

then be degraded by lysosomal enzymes, such as cathepsin K. In parallel to this process, a reduction in factors that suppress osteoblast differentiation (such as sclerostin and DKK-1) and an increase in factors that induce osteoblast differentiation (such as WNT, TGFβ and insulin-like growth factor 1 (IGF-1)) promote the formation of osteoblast pre-cursors from mesenchymal derived progenitors [33-35]. This process is tightly regulated through the master transcriptional regulator runt-related transcription factor 2 (RUNX2), mediating the expression of osteoblast specific genes such as osteocalcin, osteopontin and bone sialoprotein [36, 37]. As osteoblasts continue to mature, RANKL levels (which maintain osteoclasts) decrease, whilst osteoprotegerin (OPG) (the dummy receptor for RANKL that suppresses RANK signalling) increases. Together with increasing TGFβ signalling, the decrease in RANK/RANKL signalling leads to reduced osteoclast differentiation, activity and survival [38]. The transition toward the reversal phase is characterised by an increase in mature osteoblasts at the vacated osteoclast lacunae site of bone resorption. One key cell type that appears to facilitate this transition appears to be a unique cell population known as reversal cells, which cover the eroded bone surface. Here, one study has revealed that the disruption of these cells results in a loss of the initiation of bone resorption, highlighting their importance in this process [39]. Mature osteoblasts then secrete factors required for osteoid formation including organic matrix rich in type 1 collagen, osteocalcin and bone sialoprotein (BSP) [40]. This is then mineralized via the deposition of hydroxyapatite crystals, created by the flux of

calcium and phosphate ions within vesicles that are deposited as a mineralized nodule, in a process that has been shown to require the enzyme alkaline phosphatase to release phosphate ions [41, 42]. Ultimately, bone formation ceases as osteoblasts undergo apoptosis or are incorporated into the osteocyte network.

4.4 Regulation of bone metabolism by inflammation

In diseases such as RA, IBD and COPD, ongoing systemic inflammation results in inflammatory osteoporosis, with localized destruction of bone at sites of inflammation in diseases such as RA [43-47]. Systemic bone loss is characterized by a general decrease in bone mineral density (BMD) at the femoral neck, hip and spine in patients, resulting in increased fracture rates [47-50]. It is widely accepted that this inflammatory bone loss results from an imbalance in the bone remodelling cycle, shifting towards resorption and away from formation [51]. Studies exploring inflammatory bone loss are complicated by immobility in patients and the impact of concurrent anti-inflammatory drugs that can influence bone metabolism. However, significant insights have been derived from in vitro studies and clinical studies.

A prominent mechanism associated with a shift toward inflammatory bone loss is the interaction of the activated immune system with bone resorbing osteoclasts. Here, changes in the inflammatory cytokine profiles in patients with chronic inflammation result in increased levels of pro-osteoclastogenic mediators and a decrease in anti-osteoclastogenic mediators. Many of the pro-

osteoclastogenic cytokines upregulated in chronic inflammation, including TNF-α, IL-1, IL-6, IL-8 and IL-17, mediate their actions via an upregulation of RANKL on fibroblasts and osteoblasts, which in turn promotes osteoclastic bone resorption [52-55]. In particular, combinations of cytokines including TNF-α, IL-1 and IL-6, act synergistically to increase RANKL in inflammation [56]. Activated Th17 and B cells also upregulate RANKL expression promoting resorptive bone lesions in patients and in vitro in a RANKL dependent manner [57-59]. A recent study identified a novel cytokine induced in response to TNF-α in T cells, known as secreted osteoclastogenic factor of activated T cells (SOFAT), which has the ability to cause osteoclastogenesis in a RANKL independent manner and may have implications in bone loss induced by chronic inflammatory disease [60].

Of particular interest, TNF-α also has effects on the bone forming ability of osteoblasts in inflammation. TNF-α treatment of osteoblasts' precursors inhibits their differentiation by suppressing the DNA binding ability of RUNX2, leading to inhibition of alkaline phosphatase expression and matrix deposition [61]. The pro-apoptotic properties of TNF-α on osteoblasts has also been observed [62]. Similarly, IL-6 treatment of osteoblasts leads to reductions in alkaline phosphatase activity and in the expression of RUNX2 and osteocalcin, with mineralisation dramatically reduced in a dose dependent manner [63]. The prominent role of the inflammatory activation of osteoclastogenesis was derived from murine models using the TNF-tg mouse of chronic polyarthritis and inflammatory bone loss. Here, blockade of both

the TNF-α and the RANKL/RANK signalling pathways using anti-TNF therapy in combination with anti-osteoclastic (OPG) was able to prevent inflammatory bone erosions [64]. Bone repair was then augmented through the addition of the pro-osteoblastic hormone parathyroid hormone (PTH). These results highlight the importance of both inflammatory activation of osteoclasts and suppression of osteoblasts in mediating systemic and localized bone loss in chronic inflammation. Consequently, these results indicate that repair of bone erosions requires a therapy that simultaneously controls inflammation while also impacting both osteoclastic bone resorption and osteoblastic bone formation to shift the balance in bone homeostasis and promote normal repair and recovery of bone.

4.5 Effects of glucocorticoids on bone metabolism

Whilst GCs are widely used in the treatment of chronic inflammation, they are themselves associated with an increased risk of fractures and osteoporosis at therapeutic doses resulting in GIO. GIO is the most common form of secondary osteoporosis with risk of fracture increasing dramatically within three to six months of starting GC therapy [65]. Interestingly, these changes are reversed rapidly upon cessation of GCs, indicating a rapid and acute nature of action at the cellular level. The mechanism that underpins this appears to be primarily mediated by a substantial inhibition of osteoblastic bone formation [66]. Under physiological conditions, GCs promote osteoblast maturation. However, at higher therapeutic

doses, GCs downregulate WNT agonists and upregulate WNT inhibitors, which induce apoptosis and suppress osteoblast differentiating [67-69]. In one clinical study examining children receiving exogenous glucocorticoids, serum levels of the WNT signalling inhibitor DKK-1 were shown to be significantly elevated, suggesting it may play a key role in reduced bone formation in GIO [70]. In studies using transgenic mice with osteoblast targeted disruption of glucocorticoid signalling, GC signalling via the GR was shown to mediate reduced bone formation through the suppression of osteoblast differentiation via the WNT pathway and through inducing osteoblast apoptosis, with animals with GR signaling disruption being protected from GC induced bone loss [67, 71].

The impact of GCs on osteoclasts is less clear. Studies have reported that GC treatment results in a decrease in osteoclast number, but an increase in osteoclast longevity, potentially mediated via a GC induced increase in M-CSF production [66, 72, 73]. In addition, studies have shown conflicting results on the expression of osteoclastic genes in response to GCs. One study showed that dexamethasone treatment of murine calvarial bones resulted in increased mRNA levels of Rank and Rankl, leading to increased markers of osteoclast activation [74]. Other studies showed that OPG levels are suppressed or reported no change at all in RANKL and OPG levels [72, 75, 76]. Some insight comes from one study in children receiving exogenous GCs, where serum levels of RANKL were elevated and OPG suppressed [77]. In these patients, spontaneous osteoclastogenesis in vitro was

apparent in monocytic cell precursors. Certainly, one study utilizing a murine model of therapeutic GC delivery revealed that the targeting of osteoclasts using bisphosphonates was an effective strategy to prevent both cortical and trabecular bone loss [78]. There is some evidence to indicate that the responsiveness of osteoclasts to GCs is highly dependent on the stage of cell differentiation, but these findings require further investigation [79]. The variation in GC dose, the method of administration and the models employed may explain the variation in the results reported to date, whilst their interactions with inflammatory mediators in patients with chronic inflammation should also be taken into account when investigating their bone related effects.

4.6 Glucocorticoids, inflammation and bone homeostasis

Glucocorticoids directly oppose a wide array of the pathways that drive inflammatory bone loss. Amongst these, their suppression of pro-inflammatory factors such as RANKL, TNF-α and IL-6 appears to be prominent in mediating their bone sparing effects in chronic inflammatory joint destruction, through the direct suppression of osteoclastogenesis and osteoclast activation [80] (Figure 2). In contrast, their potent suppression of anabolic bone formation by osteoblasts may synergize with the deleterious actions of inflammation on osteoblasts. Consequently, the net balance of GCs on bone metabolism in the context of chronic inflammation is less clear. Several clinical studies shed light on the balance between

beneficial and detrimental actions of GCs on bone metabolism in chronic inflammation. These include a study reporting no differences in BMD loss in RA patients receiving therapeutic GCs in combination with traditional disease modifying anti-rheumatic drugs (DMARDS), relative to a matched control cohort [81]. Of particular interest were studies exploring whether GCs at lower therapeutic doses might promote positive anti-inflammatory actions without eliciting detrimental bone loss. These studies reported that low dose GC therapy in RA did not increase the risk of generalized osteoporosis at the spine and hip [82, 83]. Another study found that patients receiving GCs in combination with anti-TNF therapy had a 2.5% increase in BMD at the femoral neck compared to a 0.7% decrease in BMD in those using anti-TNF alone, suggesting that GCs may increase bone metabolism in this context [84]. In contrast, two further studies found that GCs' use was associated with decreased BMD in RA patients [43, 85]. Similarly, in juvenile chronic arthritis (JCA), two studies found that GC treated patients had significantly less trabecular bone and higher risk of vertebral collapse than a matched control cohort [86, 87]. These studies found strong links with the dose of steroid applied, but were further complicated by the application of GCs in the developing skeleton of younger patients, who may be more vulnerable to the anti-anabolic actions of GCs than adults. The conflicting nature of these results may stem from a variety of issues, including differences in disease pathophysiology, disease activity, duration and variations in the delivery and dose of GC therapy. In addition, concomitant use of alternative therapies such as anti-TNF

treatments causes further complications, making it difficult to dissect the contribution of GCs to changes in bone metabolism in chronic inflammatory disease.

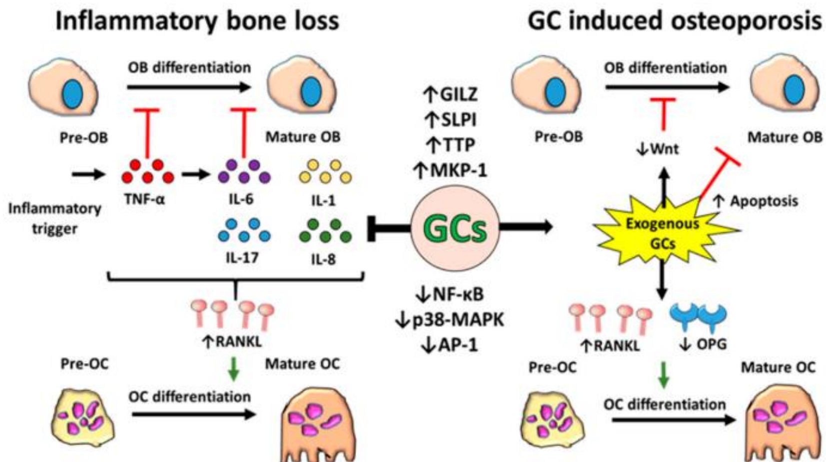

Figure 2. Schematic representation of the effects of inflammation and glucocorticoids (GCs) on bone remodelling. During inflammation, elevated levels of pro-inflammatory cytokines, such as TNF-α and IL-6, inhibit the differentiation of bone forming osteoblasts from their precursors. These cytokines, along with other pro-inflammatory mediators including IL-1, IL-17 and IL-8, also upregulate the expression of receptor activator of nuclear factor kappa-B ligand (RANKL), which binds to receptor activator of nuclear factor kappa-B (RANK) on pre-osteoclasts and triggers their differentiation into mature bone resorbing osteoclasts. Overall, bone formation is decreased while bone resorption is increased, leading to a net loss of bone. Although GCs suppress inflammation via suppression of pro-inflammatory factors and induction of anti-inflammatory mediators, they can also independently drive bone loss by inhibiting differentiation and inducing apoptosis of osteoblasts whilst increasing osteoclast differentiation by stimulating expression of RANKL and decreasing its decoy receptor osteoprotegerin (OPG). Osteoblasts (OBs), p38 mitogen-activated protein kinases (p-38-MAPK), glucocorticoid-induced leucine zipper (GILZ), secretory leukocyte protease inhibitor (SLPI), tristetraprolin (TTP), mitogen-activated protein kinase-1 (MKP-1), activator protein 1 (AP-1), OC (osteoclast), canonical WNT signalling (WNT), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).

4.7 Muscle mass related metabolism

Similar to bone, muscle metabolism is tightly regulated to ensure a balance between anabolic and catabolic processes governing muscle mass. Its regulation is critical not only to facilitate mechanical locomotion, but also as a key site for whole body energy metabolism and homeostasis [88]. Several critical anabolic and catabolic signalling pathways determine muscle protein synthesis, muscle proteolysis and myogenesis as cellular processes in control of muscle mass.

IGF-1 has been identified as a critical factor mediating the regulation of anabolic and catabolic muscle homeostasis in adult myofibers. Produced primarily in the liver, its binding to the IGF-1 receptor (IGF1R) in skeletal muscle allows recruitment of the insulin receptor substrate 1 (IRS-1) and activation of phosphatidylinositol-3-kinase (PI3K) and phosphorylation of protein kinase B (known as AKT), [89, 90]. Together, these result in the activation of the mammalian target of rapamycin (mTOR) signalling pathway, which results in suppression of proteolysis and activation of muscle protein synthesis. mTOR activation suppresses proteolytic, forkhead box class O family member proteins (FOXOs) and glycogen synthase kinase-3 beta (GSK-3β) pathways [91, 92]. The activation of mTOR signalling promotes muscle protein synthesis through the downstream phosphorylation and inactivation of eIF4E-binding protein 1 (4E-BP1) and activation of the ribosomal protein S6 kinase beta-1 (p70S6K) [93-95]. When active, 4E-BP1 operates by suppressing the eukaryotic translation initiation factors (eIF), which are a central rate limiting step in

the regulation of protein synthesis in muscle. Here, eIF4F (a complex of initiation factors, eIF4e, eIF4G and eIF4A), promotes the translation of mRNA coding for muscle proteins by facilitating the cap dependent binding of messenger RNA to the 40S ribosomal subunit [96]. The repressor protein 4E-BP1 is a powerful negative regulator of eIF4F mediated protein translation, whilst its phosphorylation causes its dissociation from eIF4E and enables mRNA translation of anabolic muscle proteins. A second key stage in the regulation of anabolic protein metabolism in muscle occurs through the regulation of phosphorylated p70S6K by mTOR, which facilitates ribosomal biogenesis and translation capacity required for muscle protein anabolism [97]. An additional modulator of skeletal muscle mass downstream of the IGF-1/AKT pathway is GSK-3β. This protein kinase is a negative regulator of the translation initiation factor eIF2B and is phosphorylated and inactivated by AKT, allowing initiation of mRNA translation [98-100]. Together, the activation of these pathways by IGF-1 or insulin promote protein synthesis in muscle, favouring increased muscle mass.

The regulation of muscle catabolism shares many of these pathways and involves their inverse activation state. Proteolysis of skeletal muscle proteins through their targeted degradation by the ubiquitin-proteasome system (UPS) and autophagy pathways is under stringent control of the PI3K/AKT and mTOR signalling pathways [101]. Here, a reduction in anabolic factors such as IGF-1 or an increase in negative regulators such as myostatin, TGFβ or FGF results in a decrease of the

PI3K/AKT and mTOR signalling. As AKT and mTOR kinase activity is responsible for inhibitory phosphorylation of the FOXOs, including FOXO1, FOXO3 and FOXO4 [102, 103], the lack thereof allows their nuclear translocation. FOXO transcription factors bind to promoter and enhancer regions of target genes such as the E3 ligases, Atrogin-1 and muscle RING-Finger protein-1 (MURF-1) and the autophagy-related genes LC3 and Bnip3 [104-107]. In addition to FOXO, increased GSK-3β secondary to reduced IGF-1/AKT signalling has also been implicated in upregulating Atrogin-1 and MURF-1 [108].

The E3 ligases are the largest family of ubiquitination factors targeting muscle proteins for degradation by the UPS [109, 110] and can be highly upregulated in catabolic conditions. These include the muscle specific F-box protein Atrogin-1 encoded by the FBXO32 gene and MURF-1 encoded by the TRIM63 gene [111, 112]. Their expression is elevated in a plethora of skeletal muscle atrophy models, including immobilisation, denervation, cancer, starvation and diabetes [111-113]. Atrogin-1 has been shown to ubiquitinate desmin and vimentin, muscle proteins essential to sarcomere Z-disk architecture [114]. In addition, Atrogin-1 stimulates the degradation of transcription factor EIF3F, leading to impaired muscle protein synthesis [115]. This E3 ligase has also been shown to play a pivotal role in repressing myogenesis through the ubiquitination of myoblast determination protein 1 (MYOD) [116].

MURF-1 encodes a protein containing a RING finger domain, which is responsible for its ubiquitin-ligase activity [112, 117]. MURF-1 ubiquitinates and catalyses the degradation of contractile proteins and thick filaments, such as myosin and troponin I, with the sparing of thin filaments such as actin [118, 119]. Besides a role in the UPS, increased FOXO activation also upregulates protein degradation and clearance through the autophagy pathways [106]. In muscle, this appears to be mediated through the direct upregulation of autophagy genes such as LC3, BNIP3 and ATG through the FOXO pathway during muscular atrophy [120-122].

Postnatal myogenesis is an anabolic process important to the maintenance of muscle mass and integrity. Insulin-like growth factor 1 (IGF-1) has been shown to be a positive driver of myogenesis, whilst fibroblast growth factor (FGF), transforming growth factor β (TGF- β) and myostatin are potent inhibitors [123-126]. In addition, various secreted WNT signalling factors positively influence myogenesis. These are regulated by an array of stimuli, including exercise, nerve innervation and dietary protein intake, and are mediated through various gene regulatory networks including the T-box family, tbx6, ripply1 and mesp-ba in mesenchymal stem cell populations [127-129]. Ultimately, this drives the expression of myogenic regulatory factors (MRFs) such as myogenic differentiation 1 (MYOD), myogenic factor 5 (MYF5) and myogenin (MYOG), this process being in mesenchymal derived muscle progenitor cells called satellite cells [130-133]. Although some redundancy exists in their cellular function, MYF5 is mostly implicated in mediating the proliferation of

satellite cells and MYOD in their differentiation into myoblasts, whilst downstream factors, including myogenin, initiate further differentiation of mature myocytes followed by the fusion and formation of mature myotubes [130-132] or mostly relevant for adult muscle, fusion with myofibers.

Below, we will describe how inflammation and glucocorticoids impact these regulatory processes of muscle mass metabolism, driving a shift towards anti-anabolic and catabolic protein metabolism, resulting in muscle wasting.

4.8 Effects of inflammation of muscle metabolism

Inflammation is a well-established driver of muscle wasting in preclinical models and strongly relates to poor prognostic outcome and increased morbidity and mortality in patients with chronic inflammatory diseases [134]. Pro-inflammatory cytokines such as TNFβα, IL-1β and IL-6, which are elevated in chronic inflammation, are themselves reported to drive proteolysis and autophagy and suppress myogenesis and protein synthesis in muscle [135-138]. Of these, TNF-α, at the apex of the inflammatory cytokine cascade in many chronic inflammatory diseases, is critical in regulating inflammatory muscle wasting. Here, its activation of the NF-kB and p-38 MAPK pathways directly induces muscle wasting through the increased expression of the E3 ligases, atrogin-1 and MURF-1 and activation of the UPS system [139-143]. In models of chronic inflammation, TNF-α also downregulates circulating levels of IGF-1 and the downstream PI3K/AKT/mTOR signalling pathways,

whilst upregulating the catabolic FOXO pathway to suppress protein synthesis and myogenesis in muscle [141, 142, 144, 145]. Another factor implicated in inflammatory muscle wasting is myostatin. This is also reported to be increased in chronic inflammation, where it positively correlates with markers of disease severity. Elevated myostatin downregulates PI3K/AKT/mTOR signalling, promoting muscle atrophy [146, 147]. Of interest, several studies have reported elevated endogenous GC levels as being central mediators of inflammatory muscle wasting. Here, the inflammatory activation of the hypothalamic/pituitary/adrenal (HPA) axis in response results in an elevation of circulating cortisol to mediate muscle wasting [148-151]. Of note, the blockade of endogenous GC production or muscle GR signalling could reverse muscle wasting in some experimental models [152, 153]. This indicates that in addition to a direct impact of inflammation on intra-cellular muscle mass regulatory processes, activation of the HPA axis as an evolutionarily conserved response to suppress systemic inflammation can result in GC driven muscle wasting as an indirect effect of inflammation on skeletal muscle.

4.9 Effects of glucocorticoids on muscle metabolism

Extended exposure to therapeutic GCs results in the rapid onset of a GC induced muscle atrophy, characterised by a decrease in myogenesis and protein synthesis and an increase in proteolysis and atrophy of muscle fibres [9, 154-157]. This leads to a significant decrease in muscle fibre size, with a greater degree of

wasting apparent in fast-twitch or type II muscle fibres [158]. The shift towards greater catabolic loss of protein and decreased anabolic synthesis in muscle is elicited by GCs through a number of pathways, including a decrease in IGF-1 signalling and an increase in negative regulators of the mTOR pathways such as myostatin and the protein regulated in development and DNA damage response 1 (REDD1) [157, 159]. Similarly, as with inflammatory pathway activation, GCs also activate the UPS and autophagy secondary to upregulation of the FOXO1 pathway [80, 154, 160]. In particular, the marked increase in degradation of contractile skeletal muscle proteins through the UPS system is believed to be central in GC induced muscle wasting in vivo. This is supported by several studies demonstrating the downregulation of the PI3K/AKT/mTOR signalling pathways and the upregulation of the E3 ligases Atrogin-1 and MURF-1 in response to GCs [112]. Several studies have also demonstrated a significant increase in 4E-BP1 and suppression of p70S6K in GC induced muscle atrophy, demonstrating a role for reduced protein synthesis and regeneration [157, 159]. Of interest, the restoration of IGF-1 signalling can rescue GC induced myopathy in mice, demonstrating a crucial role for this growth factor in the process of GC-induced myopathy [9, 154-156]. Glucocorticoid mediated muscle wasting has also been shown to be rescued through the in vivo deletion of myostatin, indicating that the negative regulation of the IGF-1 pathway may also be a crucial step in this process [161-163].

4.10 Interaction between inflammation and glucocorticoids in muscle

As with bone, many of the central inflammatory pathways that induce muscle wasting, including the NF-kB and p38-MAPK pathways, are themselves suppressed by GC signalling, suggesting that therapeutic application may protect against the process of inflammatory muscle wasting. However, other elements of inflammatory muscle wasting such as the suppression of IGF-1 and induction of myostatin and FOXO1 pathway activation are common components in both inflammatory and GC induced muscle wasting (Figure 3). Understanding how these interact in vivo remains paramount in our understanding of how therapeutic GCs should be applied in the setting of chronic inflammatory disease. Some insights arise from clinical studies exploring these processes in patients with inflammatory disease receiving GCs. Of note, in inflammatory myopathies arising directly from muscle inflammation, such as with polymyositis and dermatomyositis, GCs are effective in controlling inflammation and protecting against inflammatory muscle wasting and associated weakness [164]. Similarly, therapeutic GCs are effective in preventing muscle wasting in patients with Duchenne muscular dystrophy (DMD), where progressive muscle necrosis mediates loss of muscle [165-169]. However, in other inflammatory diseases, where muscle wasting occurs secondarily to inflammation at a non-muscle site, the application of therapeutic GCs is strongly associated with rapid loss of muscle mass [170, 171]. These findings may suggest that GCs can oppose the process of inflammatory muscle wasting when the active inflammation is

confined to the muscle, but promote muscle wasting when used to manage other systemic chronic inflammatory diseases such as RA. As with GC induced osteoporosis in patients with chronic inflammatory disease, the interpretation of these findings in relation to muscle wasting is complicated by disease severity and duration and by concurrent DMARD therapies.

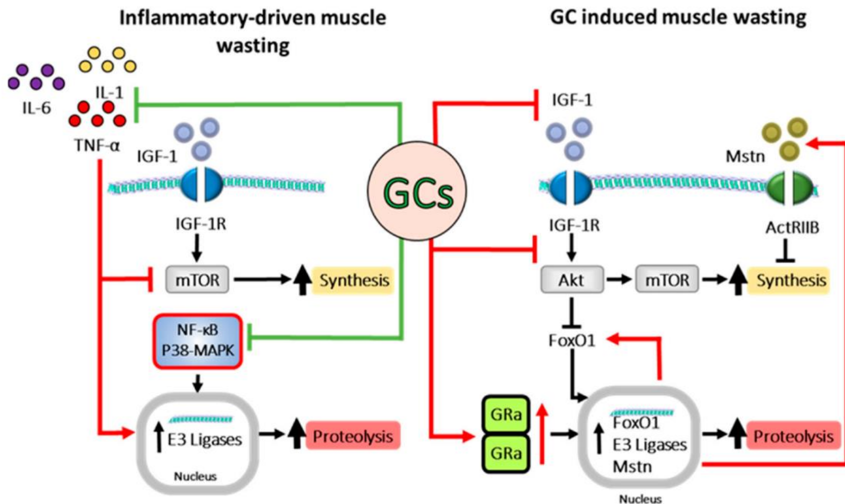

Figure 3. Schematic representation of signalling pathways involved in both inflammatory driven and GC induced muscle wasting and their interactions. Inflammatory cytokines such as TNF-α and IL-1 inhibit mammalian target of rapamycin (mTOR) signalling, dampening muscle protein synthesis, whilst simultaneously inducing transcription of E3 ligases, leading to muscle proteolysis. Glucocorticoids (GCs) inhibit inflammatory signalling, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling, and therefore decrease inflammatory driven muscle wasting. Despite this, GCs also drive muscle wasting through several pathways themselves, including suppression of the IGF-1/AKT/mTOR signalling cascade, leading to decreased protein synthesis and increased FOXO1 transcription. GR activation and dimerization induce the transcription of myostatin (MSTN), FOXO1 and other E3 ligases, leading to increased proteolysis and diminished protein synthesis. Forkhead box protein O1 (FOXO1), mammalian target of rapamycin (mTOR), insulin like growth factor (IGF-1), (p-38-MAPK), glucocorticoid induced leucine zipper (GILZ), secretory leukocyte protease inhibitor (SLPI), tristetraprolin (TTP), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), glucocorticoid receptor (GR), myostatin (MSTN), and IGF-1 receptor (IGF-1 R).

4.11 Insights from murine models of chronic inflammation receiving therapeutic glucocorticoids

Additional insight has come from murine models of polyarthritis receiving therapeutic GCs. These are able to circumvent issues related to differences in disease activity between patients and complications arising from the various alternative anti-inflammatory drugs used to manage disease in patients. In one such study, we examined the role of the GC corticosterone, delivered as a monotherapy in the TNF-tg murine model of polyarthritis, on net bone and muscle metabolism [80]. This revealed that therapeutic doses of GCs, whilst effective at suppressing disease activity, also potently suppressed inflammatory osteoporosis and juxta articular bone loss. This confirmed that their capacity to suppress inflammatory pathways that mediate inflammatory bone loss outweighed their deleterious effects on bone metabolism. These bone sparing effects of GCs were mediated through the suppression of pro-inflammatory osteoclasts' activation, both systemically and at sites of inflammation. However, whilst these treatments protected from inflammatory bone loss, we still observed a suppression of anabolic bone formation in all mice receiving GCs, suggesting that long term administration may still ultimately result in GIO.

Unlike bone, therapeutic GCs markedly exacerbated muscle wasting in mice with chronic inflammation. This was characterized by a marked activation of the catabolic FOXO1 and UPS pathways [80]. Similar findings had been reported in rats,

where dexamethasone exacerbated inflammatory muscle wasting in models of sepsis [172]. These data indicate that the beneficial effects of inflammatory suppression by GCs in muscle were not sufficient to outweigh their deleterious actions on muscle metabolism. Whilst further work is required to better elucidate the actions of therapeutic GCs in the context of chronic inflammation, these data shed light on the potential strengths and weaknesses of their application in muscle and bone. In particular, they indicate that the management of side effects in muscle may need to be prioritised over those in bone, in patients with chronic inflammatory diseases receiving therapeutic GCs.

4.12 Pre-receptor regulation of therapeutic gc action to protect against side effects

The pre-receptor metabolism of GCs by the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes is recognised as a critical step in mediating GC signalling in many peripheral tissues. These are the 11β-HSD type 1 (11β-HSD1) and type 2 (11β-HSD2). 11β-HSD1 is expressed in many tissues, including the liver, bone, muscle and fat, where it converts inactive endogenous and therapeutic GCs (such as corticosterone and prednisone) to their active counterparts (such as cortisol and prednisolone), leading to a local accumulation and concentration of active GCs [173-175] (Figure 4). In contrast, 11β-HSD2 solely inactivates endogenous and therapeutic GCs within the kidney, providing circulating inactive GC substrate for the peripheral

11β-HSD1 enzyme and supporting renal clearance of GCs [175]. Several key studies have demonstrated a critical role for the pre-receptor activation of GCs by 11β-HSD1 in mediating the deleterious actions of therapeutic GCs in muscle and bone [176, 177]. Here, animals with transgenic deletion of 11β-HSD1 are resistant to both exogenous GC induced muscle wasting and osteoporosis. This raises the exciting possibility that therapeutic 11β-HSD1 inhibitors, widely explored in the management of metabolic disease, may prevent bone loss and muscle wasting in patients with chronic inflammatory diseases receiving GCs [178, 179]. Further studies lend strength to this concept, showing that 11β-HSD1 is potently upregulated within muscle cells and osteoblasts, where it is potently upregulated by circulating inflammatory cytokines such as TNF-α and IL-1β [174, 180, 181]. Despite this, caution should be applied in this context, given that systemic deletion of 11β-HSD1 can exacerbate disease activity in murine models of inflammation, secondary to a reduction in reactivation of endogenous GC at sites of inflammation [182, 183]. Consequently, further studies are required to delineate the potential benefits and risks of 11β-HSD1 inhibition in chronic inflammatory disease.

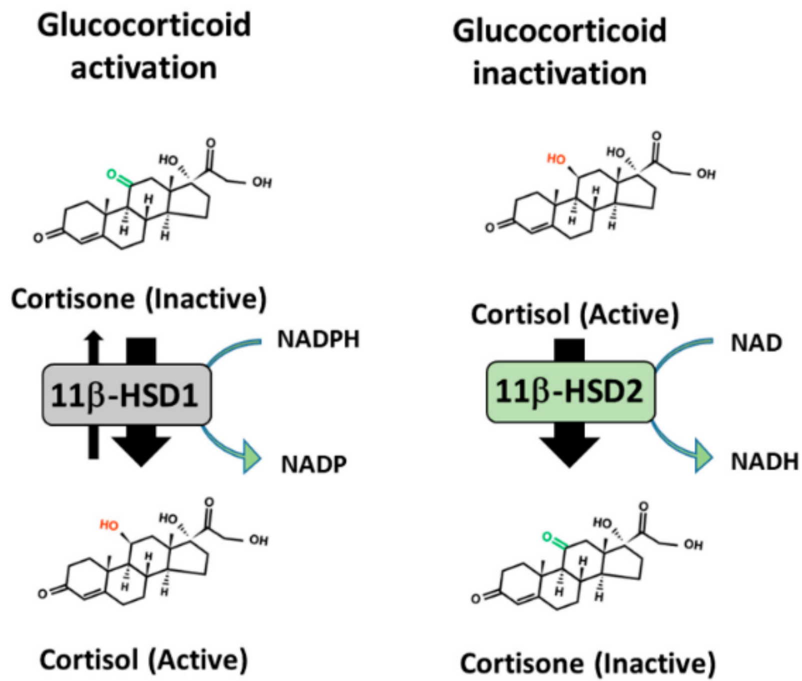

Figure 4. Pre-receptor metabolism of GCs by 11β-HSD1. 11β-hydroxysteroid dehydrogenase (11β-HSD) type 1 is a bidirectional enzyme that predominantly reduces inactive GCs to their active counterparts in an NADPH dependent manner, whilst 11β-HSD type 2 is an NAD+ dependent unidirectional enzyme that converts active GCs to their inactive counterparts.

4.13 Conclusions

Both chronic inflammation and therapeutic GCs are potent drivers for systemic bone and muscle wasting resulting from an imbalance between anabolic and catabolic homeostasis. Whilst therapeutic GCs oppose many of the inflammatory pathways that drive bone and muscle wasting, they share common pathways that promote anti-anabolic and catabolic metabolism of bone and muscle

and can drive or exacerbate these deleterious processes in chronic inflammatory disease. However, these relationships are invariably complicated by the nature of the inflammatory disease in which therapeutic GCs are utilized. Intriguingly, 11β-HSD1 inhibitors may possess the potential to prevent the deleterious actions of therapeutic GCs in the backdrop of chronic inflammation. However, further studies are required to assess their efficacy and safety in this context.

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Conflicts of interest

The authors declare no conflict of interest.

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Chapter 5 – Therapeutic glucocorticoids prevent bone loss but drive muscle wasting when administered in chronic polyarthritis

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Abstract

Background

Patients with rheumatoid arthritis (RA) experience extra-articular manifestations including osteoporosis and muscle wasting, which closely associate with severity of disease. Whilst therapeutic glucocorticoids (GCs) reduce inflammation in RA, their actions on muscle and bone metabolism in the context of chronic inflammation remain unclear. We utilised the TNF-tg model of chronic polyarthritis to ascertain the impact of therapeutic GCs on bone and muscle homeostasis in the context of systemic inflammation.

Methods

TNF-tg and wild-type (WT) animals received either vehicle or the GC corticosterone (100 µg/ml) in drinking water at onset of arthritis. Arthritis severity and clinical parameters were measured, serum collected for ELISA and muscle and bone biopsies collected for µCT, histology and mRNA analysis. In vivo findings were examined in primary cultures of osteoblasts, osteoclasts and myotubes.

Results

TNF-tg mice receiving GCs showed protection from inflammatory bone loss, characterised by a reduction in serum markers of bone resorption, osteoclast numbers and osteoclast activity. In contrast, muscle wasting was markedly increased in WT and TNF-tg animals receiving GCs, independently of inflammation. This was

characterised by a reduction in muscle weight and fibre size, and an induction in anti-anabolic and catabolic signalling.

Conclusions

This study demonstrates that when given in early onset chronic polyarthritis, oral GCs partially protect against inflammatory bone loss, but induce marked muscle wasting. These results suggest that in patients with inflammatory arthritis receiving GCs, the development of interventions to manage deleterious side effects in muscle should be prioritised.

5.1 Introduction

Patients with inflammatory arthritis experience extra-articular manifestations, including osteoporosis and muscle wasting, which closely correlate with measures of disease activity [1-4]. Glucocorticoids (GCs) are effective at controlling inflammation in rheumatoid arthritis (RA) and are recommended as an initial line of therapy for the rapid control of disease [5-7]. However, long-term GC use is associated with osteoporosis and systemic muscle wasting, resulting in increased fracture risk and mortality in patients with RA [8-13].

It remains unclear what the effects of GCs on bone and muscle are, when used to treat new onset inflammatory arthritis, in particular, whether the beneficial effects of controlling articular and systemic inflammation on bone and muscle outweigh their direct catabolic actions in these tissues.

Murine models of polyarthritis have proven a powerful tool in examining the pathophysiology of inflammatory diseases, such as RA. The TNF-tg mouse is a murine model of polyarthritis driven by the transgenic overexpression of the pro-inflammatory cytokine TNFα that proved valuable in the initial validation of anti-TNFα biologicals [14]. We have previously shown that this animal model develops systemic bone loss and muscle wasting in a manner consistent with human disease [15, 16]. In this study, we use the TNF-tg model of polyarthritis and wild-type (WT) counterparts to examine the effects of orally administered anti-inflammatory GCs on bone and muscle metabolism in the context of systemic inflammation. We

demonstrate that during active polyarthritis, therapeutic GCs are effective at suppressing synovitis, joint destruction and systemic bone loss, but markedly promote systemic muscle wasting.

5.2 Materials and methods

5.2.1 TNF-transgenic mouse model

Procedures on animals were performed under guidelines by the Animal (Scientific Procedures) Act 1986 in accordance with the project licence (P51102987) and approved by the Birmingham Ethical Review Subcommittee (BERSC). The TNF-tg model of chronic inflammatory polyarthritis, obtained courtesy of Dr. George Kollias (BSRC Fleming, Athens), were maintained on a C57BL/6 background and compared to WT littermates [17]. At day 32 of age, at the first onset of measurable polyarthritis, male TNF-tg mice received drinking water supplemented with either corticosterone (Cort) (100 µg/mL, 0.66% ethanol), or vehicle (0.66% ethanol) for 3 weeks. Interventions were designed to model initial preventative bridging therapeutic glucocorticoid treatments in early onset disease. Following the administration of vehicle and corticosterone, mice were scored twice weekly for clinical scores of disease activity and arthritic paw scores as previously described [18, 19]. Mobility of animals within cages was assessed by measuring numbers of rotations walked by animals in a 3-min period and normalised to rotations per minute to get an activity score. At day 53, serum was collected by cardiac puncture under terminal

anaesthesia, and tissues excised for analysis. Wet tissue weights (mg) of tibialis anterior, quadriceps and tibia were recorded, normalised to total body weights and either snap frozen of fixed in 4% formalin (mg).

5.2.2 Primary human osteoblast culture

Following ethical approval (UK National Research Ethics Committee 14/ES/1044), patients with hip osteoarthritis (OA) (age 69 ± 3 years, Kellgren Lawrence grade 3/4; *n* = 4) were recruited prior to elective joint replacement surgery. Trabecular chips of approximately 400–600 mg were excised and placed in PBS prior to culturing. Reagents were obtained from Sigma (Gillingham, UK) unless otherwise stated. Trabecular bone chips from patient samples were cultured in osteoclast growth media to facilitate release of osteoblasts (Additional file 1: Table S1). Osteoblasts were allowed to grow and once confluent bone chips were removed. Osteoblasts were then differentiated in media containing TNFα (10 ng/ml) and/or cortisol (1000 ng/ml). Treatments were replaced three times per week. Cultures were stained with 0.5% alizarin red S to confirm differentiation into mature osteoblasts.

5.2.3 Primary human osteoclast culture

Peripheral blood mononuclear cells (PBMCs) from healthy donors, obtained from the Scottish National Blood Transfusion Service (approved by Glasgow NHS

Trust-East Ethics Committee), were isolated via Ficoll-paque PLUS (GE Healthcare) density gradient centrifugation and $CD14^+$ monocytes isolated using positive selection (Miltenyi). Monocytes were cultured in selective survival media (Additional file 1: Table S1). Osteoclasts were generated using supplementation with1ng/ml RANKL over 72 h before stimulation with vehicle, 10 ng/ml TNFα or 1000 ng/ml corticosterone (or DMSO vehicle) as appropriate. Osteoclast numbers were assessed by staining with tartrate-resistant acid phosphatase (TRAP) kit (Sigma-Aldrich). Osteoclast activity was assessed on mineral-coated plates (Corning) at day 14. Images were acquired using EVOS FL Auto Cell Imaging System (Life Technologies). Osteoclasts were identified as TRAP +ve multinucleated cells (nuclei ≥ 3). Resorption area was calculated using Fiji software (ImageJ) and defined as % resorbed area of entire well.

5.2.4 Primary murine muscle cell culture

Primary myotubes were generated from tibialis anterior as previously described [20]. In brief, whole tibialis anterior muscle was removed from WT C57/Bl6 animals at 9 weeks and digested in type 1 collagenase at 37 °C for 2 h before isolation of individual fibres. Fibres were plated in 2 ml of muscle expansion medium (Additional file 1: Table S1) and grown in plates coated with Matrigel™ (Corning Life Sciences, Flintshire, UK) (diluted 1/40 in DMEM High Glucose). Satellite cells migrating from muscle fibres were removed and cultured in maintenance medium

until confluent, prior to differentiation in selective media for 5 days (Additional file 1: Table S1).

5.2.5 *Gene expression analysis*

Gene expression in cells and tissues was assessed using TaqMan® Gene Expression Assays (ThermoFisher Scientific). Tissues were homogenised in liquid nitrogen with a sterile pestle and mortar. mRNA was isolated using an innuPREP RNA Mini Kit (Analytikjena, Cambridge) as per the manufacturer's instructions. One microgramme of RNA per sample was reverse transcribed using Multiscribe™ using the manufacturer's protocol (ThermoFisher Scientific) to generate cDNA. Alp, Bglap, Redd1, Foxo1, Trim63 and Fbxo32 were determined using species-specific probe sets by real-time PCR on an ABI7500 system (Applied Biosystems, Warrington, UK). Final reactions are listed in Additional file 1: Table S2. mRNA abundance was normalised to that of 18S or GAPDH. Data were obtained as Ct values and ΔCt determined (Ct target – Ct 18S/GAPDH). Data were expressed as arbitrary units (AU) using the following transformation: [arbitrary units (AU) = 1000 × (2−Δct)].

5.2.6 *ELISA analysis*

Serum IL-6 (R&D Systems, Abingdon, UK), P1NP and CTX-1 (Immunodiagnostic Systems, Tyne & Wear, UK) and conditioned media pro-collagen

I α1 (R&D Systems, Abingdon, UK) were determined using a commercially available ELISA assays in accordance with the manufacturer's instructions.

5.2.7 Histological analysis of joints and muscle

Histochemistry was performed on paraffin-embedded 10-μm sections of hind paws and quadriceps of WT and TNF-Tg animals following staining with haematoxylin and eosin. Pannus size at the metatarsal-phalangeal joint interface was determined using Image J software as previously reported [18]. Sections were deparaffinised and incubated in TRAP buffer (Additional file 1: Table S3) for 1 h at 37 °C to detect osteoclasts. Quantification of osteoclast numbers on the bone surface pannus interface of the ulna/humerus joint interface were normalised to bone surface area determined by image J analysis of TRAP-stained paraffin-embedded sections. Sections were stained with H&E prior to quantitative analysis in order to visualise pannus formation at the ankle joints and CSA of fibres. For all quantifications, the mean of data from three adjacent 10-μm sections cut from the centre of the joint or from the vastus medialis from six animals was utilised and assessed using Image J software.

5.2.8 MicroCT morphometry analysis

Front paws and tibias from mice were imaged using a Skyscan 1172 micro-CT scanner (Bruker) using X-ray beam settings of 60 kV/167 μA with a 0.5-mm

aluminium filter. Projections were taken every 0.45° at 580-ms exposure. Image volumes were reconstructed using the Feldkamp algorithm (NRecon 1.6.1.5, Bruker) having applied beam hardening correction. Trabecular bone parameters (bone volume to tissue volume (BV/TV), trabecular thickness (Tb.Th) and trabecular number (Tb.N)) of the tibia were analysed using CTAn software. One millimetre of bone (150 sections) in the metaphyseal region beneath the growth plate was analysed, and regions of interest (ROI) were selected by drawing around the trabecular network for each cross-sectional slice. Front paws were reconstructed, and MeshLab 1.3.2 was used to generate meshes which could then be scored for bone erosions as described previously [18].

5.2.9 Immunoblot analysis

Briefly, muscle were homogenised in 10-fold volume excess of ice-cold sucrose lysis buffer (Additional file 1: Table S3). Protein concentration was determined using the Bradford protein assay (ThermoScientific). Forty microgrammes of protein was loaded into 4–12% Bis-Tris midi protein gels (Invitrogen) prior to electrophoresis. Proteins were transferred and blocked in blocking buffer (Additional file 1: Table S3) before incubation with primary antibodies (Additional file 1: Table S4) overnight at 4 °C. Membranes were then incubated in horseradish peroxidase-conjugated secondary antibody (1/10,000) at room temperature for 1 h. Antibody detection was performed via enhanced

chemiluminescence horseradish peroxidase substrate detection kit (Millipore). Imaging was undertaken using a G:Box Chemi-XR5 (Syngene) and band quantification via (ImageJ). All data were corrected for protein loading as determined after Ponceau S staining (Sigma-Aldrich).

5.2.10 Statistical analysis

Statistical significance was defined as $P < 0.05$ (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) using either an unpaired Student's *t* test or two way ANOVA with a Bonferroni correction Tukey post hoc analysis where a Gaussian distribution is identified.

5.3 Results

5.3.1 Oral GCs suppress disease activity in TNF-tg animals

TNF-tg mice received drinking water containing vehicle or corticosterone at 100 µg/ml for 3 weeks. Daily oral water intake of corticosterone was calculated per mouse and was shown to be 22.0 ± 0.83 and 23.2 ± 2.0 µg/g body weight/day and did not vary significantly between groups (Additional file 2: Figure S1a). Serum corticosterone was shown to be significantly elevated in both WT and TNF-tg animals receiving corticosterone following oral intake relative to vehicle-treated controls (WT vehicle, 128.3 ± 66.7, WT CORT, 456.2 ± 82.5 ng/ml; $P < 0.005$; TNF-tg vehicle, 119.8 ± 29.9, TNF-tg CORT, 485.1 ± 43.7 ng/ml; $P < 0.005$) (Additional file 2: Figure

S1b). Body weights did not vary significantly between groups (Additional file 2: Figure S1c). TNF-tg mice developed significant joint inflammation by day 53, characterised by increased joint deformity, redness and reduced mobility (clinical score, wild type, 0.6 ± 0.004 vs TNF-tg, 6.2 ± 0.3; $P < 0.005$; joint inflammation, wild type, 0.16 ± 0.0001 vs TNF-tg, 7.2 ± 0.7; $P < 0.0001$) (Fig. 1a, b). Corticosterone significantly reduced joint inflammation in TNF-tg animals (clinical score, TNF-tg/vehicle, 6.2 ± 0.3 vs TNF-tg/cort, 3.3 ± 0.9; $P < 0.005$; joint inflammation, TNF-tg/vehicle, 6.2 ± 0.3 vs TNF-tg/cort, 2.2 ± 0.4; $P < 0.0005$) (Fig. 1a, b) [21]. Scoring of synovitis and joint erosions by histology and micro-CT revealed a marked increase in vehicle-treated TNF-tg mice relative to WT controls (Fig. 1c, d, e, g). These were significantly abrogated in TNF-tg animals receiving corticosterone (joint erosion score, TNF-tg/vehicle, 13.6 ± 1.3 vs TNF-tg/cort, 5.3 ± 2.1; $P < 0.0005$, Pannus area, TNF-tg/vehicle, 0.14 ± 3.9 vs TNF-tg/cort, 0.025 ± 0.004; $P < 0.0005$) (Fig. 1c, d, e, g). Juxta articular bone loss was characterised by increased osteoclast numbers at the pannus/subchondral bone interface (Fig 1f, h). Corticosterone treatment reversed this, dramatically reducing osteoclast numbers (TNF-tg/vehicle, 18 ± 3.3 vs TNF-tg/cort, 1.5 ± 0.2; $P < 0.0005$). Serum IL-6 was potently upregulated in vehicle-treated TNF-tg animals and strongly suppressed in animals receiving corticosterone. These data demonstrate that corticosterone administered at 100 μg/ml in drinking water over 3 weeks is sufficient to markedly suppress disease activity and joint destruction.

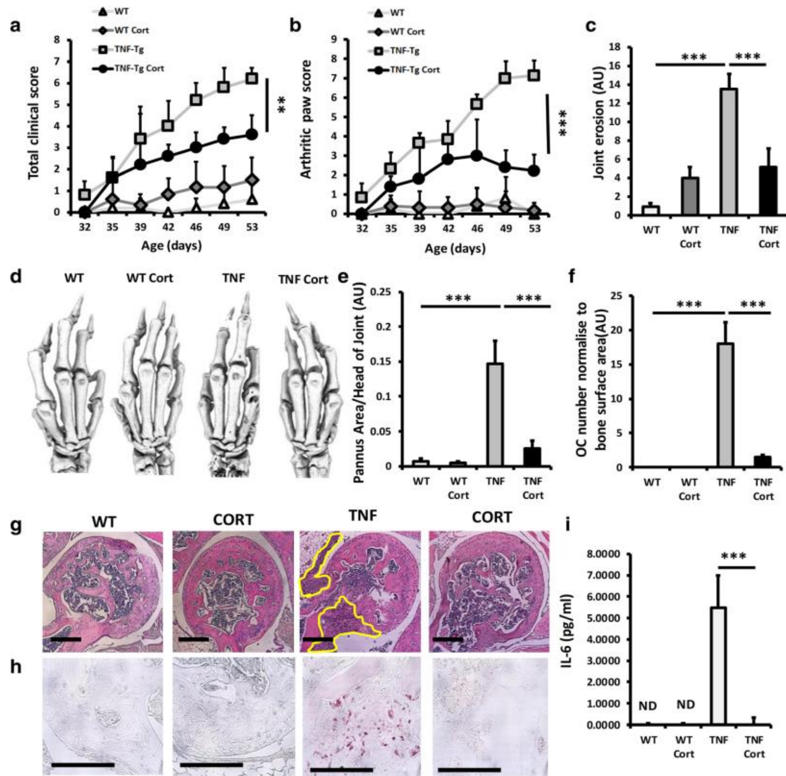

Figure 1. a Clinical scoring (weight, inflammation, grimace, behaviour, mobility, inflammation severity and duration); **b** scoring of joint inflammation; **c** quantification of cortical erosion (arbitrary units) in the bones of the ankle, metatarsals and phalanges; **d** representative images of 3D reconstructions of hind paws using micro-CT; **e** histological scoring of synovitis (arbitrary units); **f** histological scoring (arbitrary units) of TRAP +ve osteoclast numbers at the ulna/humerus joint interface; **g** representative images of synovitis at the ulna/humerus joint interface; **h** representative images of TRAP +ve osteoclast numbers at the ulna/humerus joint interface; and **i** serum IL-6 levels determined by ELISA in WT and TNF-tg animals receiving either vehicle or corticosterone (100 µg/mL) in drinking water over 3 weeks. Values are expressed as mean ± standard error of six animals per group. Statistical significance was determined using two-way ANOVA with a Tukey post hoc analysis. Black arrows indicate sites of full-thickness cortical erosions. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$

5.3.2 Oral GCs prevent trabecular bone loss during polyarthritis

In vehicle-treated TNF-tg animals, significant trabecular bone loss was apparent at day 53 (Fig. 2a). This appeared to be partially abrogated in TNF-tg animal receiving corticosterone. Analysis of trabecular bone volume to tissue volume (BV/TV), trabecular thickness (Tb.Th) and trabecular number (Tb.N) was performed in all groups. In vehicle-treated TNF-tg animals, a significant reduction in all parameters was apparent. Treatment with corticosterone partially protected from the loss in BV/TV (BV/TV: TNF-tg/vehicle, 2.1% ± 0.21 vs TNF-tg/corticosterone, 4.3% ± 0.23, $P < 0.05$) (Fig. 2b). Analysis of Tb.Th in TNF-tg animals revealed a similar loss of trabecular thickness in those treated with either vehicle or corticosterone (Tb.Th: TNF-tg/vehicle, 50.2 µm ± 3.7 vs TNF-tg/corticosterone, 50.6 µm ± 2.7, NS) (Fig. 2c). In contrast, corticosterone was able to protect against the reduction in trabecular number in this model of inflammatory polyarthritis (Tb.N: TNF-tg/vehicle, 0.0004 1/µm ± 0.00002 vs TNF-tg/corticosterone, 0.00083 1/µm ± 0.00002, $P < 0.0001$) (Fig. 2d). Together these data demonstrate that oral administration of corticosterone provides partial protection from inflammatory bone loss in TNF-tg mice, characterised by preservation of trabecular number but not thickness.

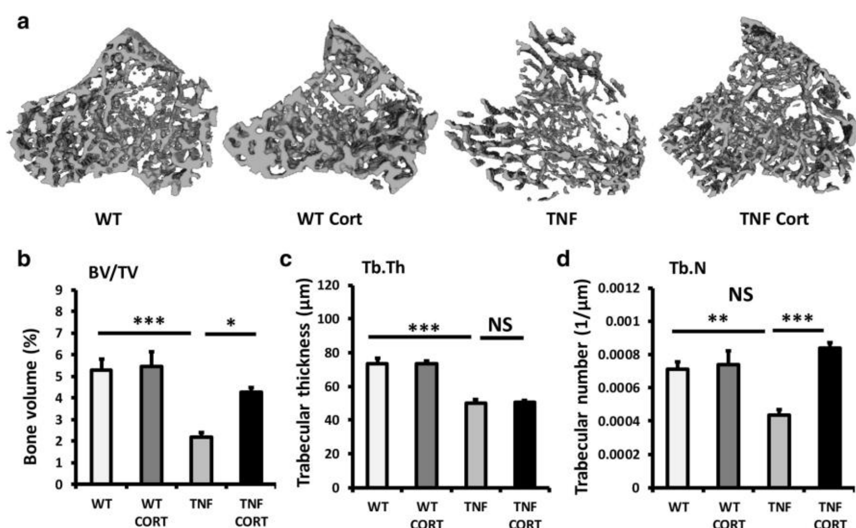

Figure *2*. a Representative images of 3D reconstructions of tibia trabecular bone using micro-CT, **b** bone volume to tissue volume (BV/TV), **c** trabecular thickness (Tb.Th) and **d** trabecular number (Tb.N) determined by Meshlab software analysis of micro-CT in WT and TNF-tg animals receiving either vehicle or corticosterone. Values are expressed as mean ± standard error of six animals per group. Statistical significance was determined using two-way ANOVA with a Tukey post hoc analysis. *P < 0.05, **P < 0.005, ***P < 0.001. Black arrows indicate erosions.

5.3.3 <u>GCs suppress both bone formation and resorption during inflammation</u>

To delineate the actions of corticosterone on bone turnover in TNF-tg mice, we examined systemic markers of bone formation (P1NP) and resorption (CTX-1) and modelled therapeutic GC treatments in human cultures of osteoblasts and osteoclasts in combination with TNFα. Whilst TNF-tg animals had significantly lower P1NP levels at day 53 relative to WT counterparts, both groups developed a comparable suppression of P1NP in response to corticosterone (wild type/vehicle,

494 ng/ml ± 46.3 vs wild type/corticosterone, 31.3 ng/ml ± 8.2; $P < 0.0001$, TNF-tg/vehicle, 269.7 ng/ml ± 27.2 vs TNF-tg/corticosterone, 32.3 ng/ml ± 7.5; $P < 0.0001$) (Fig. 3a). Analysis of mature osteoblast markers in tibia homogenates supported these data. Here, whilst gene expression of alkaline phosphatase (Alp) and osteocalcin (Bglap) were significantly reduced in TNF-tg animals at day 53 relative to WT counterparts (Alp, 2.2-fold; Bglap, 2.6-fold; $P < 0.0001$), a comparable suppression of gene expression was apparent in both groups receiving corticosterone relative to vehicle (wild type, 32-fold; $P < 0.0001$, TNF-tg, 6-fold; $P < 0.0001$) (Fig. 3b, c). In mature primary human osteoblasts, incubation with the pro-inflammatory cytokine TNFα resulted in a significant reduction in both pro-collagen production and osteocalcin mRNA (Fig. 3d, f). Here, the addition of the GC cortisol resulted in a comparable and dramatic suppression of osteoblast matrix formation and mRNA expression in control and TNFα-treated osteoblasts (pro-collagen Iα1, control, 524 ng/ml ± 128.9 vs cortisol, 50.0 ng/ml ± 93.1, $P < 0.001$, TNFα, 158.2 ng/ml ± 131.4 vs TNFα/cortisol, 11.3 ng/ml ± 6.8; $P < 0.001$; BGLAP, control vs cortisol, 43-fold suppression; $P < 0.001$, TNFα vs TNFα/cortisol; 10-fold suppression; $P < 0.05$).

Serum CTX-1 levels were determined as a measure of osteoclastic bone resorption. TNF-tg animals receiving corticosterone had a significant suppression of CTX-1 at day 53 (TNF-tg/vehicle, 87.6 ng/ml ± 11.3 vs TNF-tg/corticosterone, 36.3 ng/ml ± 3.1; $P < 0.05$) (Fig. 3g). In primary human osteoclasts, the addition of

cortisol to TNFα-stimulated cultures resulted in a significant suppression of both osteoclast numbers and calcified matrix resorption in vitro (osteoclast no., TNFα, 297.5 cells per well ± 53.7 vs TNFα/cortisol, 85.6 cells per well ± 17.8; $P < 0.05$; resorbed area, TNFα, 32.5% ± 7.0 vs TNFα/cortisol, 2.1% ± 0.8; $P < 0.05$) (Fig. 3h, i). The resorption pits in wells treated with cortisol were characterised by a reduction in both number and size (Additional file 3: Figure S2a). These data indicate that GCs suppress both osteoblast bone formation and osteoclast maturation and activity.

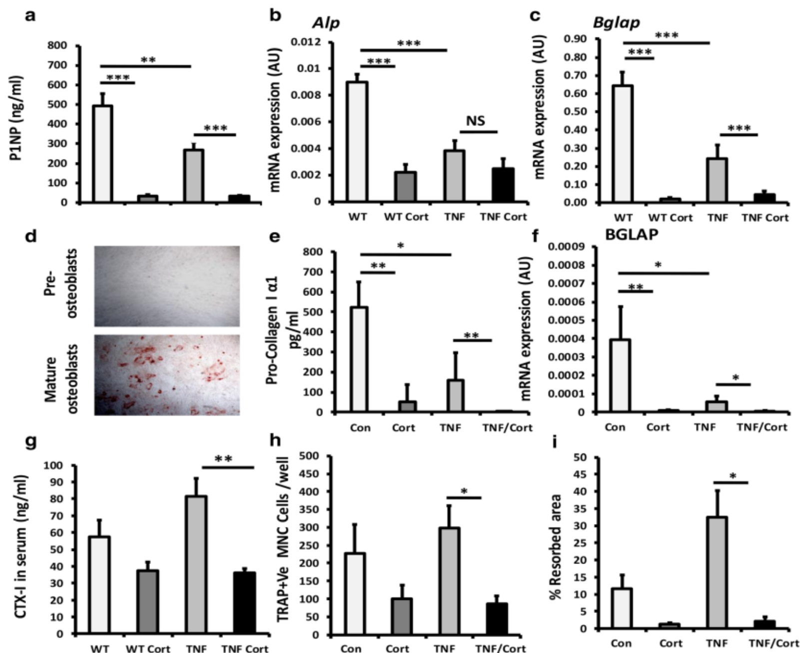

Figure *3*. **a** Serum P1NP (ng/ml) determined by ELISA. **b**, **c** Gene expression (AU) of Alp and Bglap, determined by RT qPCR in tibia homogenates, in WT and TNF-tg animals receiving either vehicle or corticosterone (100 µg/mL) in drinking water over 3 weeks. **d** Representative image of primary human pre-osteoblasts and mature nodule forming osteoblasts in vitro stained with alizarin red. **e** Pro-collagen 1 α1 formation determined by ELISA and **f** quantification of gene expression (arbitrary units) of Bglap, determined by RT qPCR in primary cultures of mature osteoblasts treated with either vehicle, TNFα (10 ng/ml), corticosterone (1 µmol/l) or a combination of TNF and corticosterone for 48 h. **g** Serum CTX-1 (ng/ml) determined by ELISA in WT and TNF-tg animals receiving either vehicle or corticosterone. **h** TRAP +ve cells per well and **i** % calcified matrix resorption in primary human osteoclasts cultures treated with either vehicle, TNFα (10 ng/ml), corticosterone (1 µmol/l) or a combination of TNFα and corticosterone over differentiation from mononuclear cells. Values are expressed as mean ± standard error of six animals or primary cultures derived from six separate individuals. Statistical significance was determined using either two-way or one-way ANOVA with Tukey post hoc analysis. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

5.3.4 *Oral GCs drive severe muscle wasting and reduce mobility in TNF-tg animals*

We examined muscle weights and morphology in WT and TNF-tg animals receiving corticosterone. Corticosterone significantly reduced quadriceps and tibialis anterior weights in WT and TNF-tg animals relative to vehicle controls (quadriceps, wild type/vehicle, 0.0029 mg/body weight ± 0.00024 vs wild type/corticosterone, 0.0019 mg/body weight ± 0.00021; $P < 0.0001$; TNF-tg/vehicle, 0.0025.7 mg/body weight ± 0.00025 vs TNF-tg/corticosterone, 0.0017 mg/body weight ± 0.00028; $P < 0.001$) (Fig. 4a, b). Analysis of animal mobility with cages was assessed at day 53 to determine the effects of polyarthritis and corticosterone treatment (Fig. 4c). Here, a significant reduction in movement was apparent in TNF-tg animals relative to WT counterparts. This was mirrored by a comparable reduction in movement seen in both WT and TNF-tg animals receiving corticosterone relative to vehicle-treated WT counterparts. Analysis of average muscle fibre cross-sectional area (CSA) indicated that this was underpinned by a reduction in muscle fibre size in WT and TNF-tg animals receiving corticosterone (fibre size, wild type/vehicle, 2064 μm^2 ± 144 vs wild type/corticosterone, 1636 μm^2 ± 96; $P < 0.05$; TNF-tg/vehicle, 1767 μm^2 ± 76 vs TNF-tg/corticosterone, 1559 μm^2 ± 88; $P < 0.05$) (Fig. 4c–e). Further analysis of fibre CSA distribution identified a significant shift towards increased small diameter fibres in TNF-tg animals relative to WT counterparts (Fig. 4e). In response to corticosterone, both WT and TNF-tg animals demonstrated a further shift in fibre CSA distribution, favouring a significant increase

in small fibres (800–1800 µm²) and significant reduction in large fibres (2200–2600 µm²) relative to vehicle-treated controls (Fig. 4f, g). In contrast, no significant shift was observed between WT and TNF-tg animals receiving corticosterone (Fig 4h). These data demonstrate that during active polyarthritis, administration of oral corticosterone promotes muscle wasting independent of inflammation.

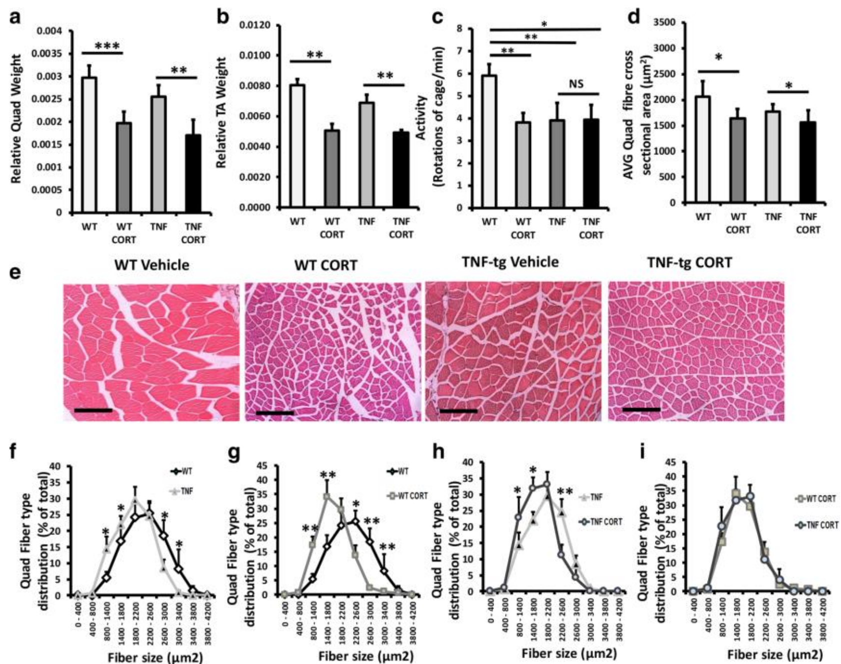

Figure 4. **a**, **b** Total quadriceps and tibialis anterior muscle weights relative to total bodyweight, **c** mouse activity determined by rotations of cage per minute, **d** average quadriceps muscle fibre cross sectional area (µm²), **e** representative images of quadriceps muscle sections, **f**–**i** distribution of quadriceps muscle fibre cross-sectional area determined using Image J in paraffin embedded sections in WT and TNF-Tg animals receiving either vehicle or corticosterone (100 µg/mL) in drinking water over 3 weeks. Values are expressed as mean ± standard error of six animals per group. Statistical significance was determined using two-way ANOVA with a Tukey post hoc analysis. *P < 0.05, **P < 0.005, ***P < 0.001 (scale bars, 50 µm).

5.3.5 *During inflammation, oral GCs drive catabolic and anti-anabolic muscle wasting*

To ascertain the pathways that underpin increased muscle wasting in TNF-tg mice treated with GCs, we examined well-defined catabolic and anti-anabolic signalling pathways in tibialis anterior muscle homogenates and in primary muscle cultures. In wild-type animals, corticosterone resulted in a comparable induction in expression of the anti-anabolic gene Redd1 and the catabolic genes Foxo1, Trim63 and Fbxo32 (Redd1, 7.7-fold; $P < 0.005$; Foxo1, 10.3-fold; $P < 0.0005$; Trim63, 7.8-fold; $P < 0.05$; Fbxo32, 8.9-fold; $P < 0.0005$) (Fig. 5a–d). Comparable inductions in gene expression of Foxo1, Trim63 and Fxo32 were also apparent in TNF-tg animals receiving corticosterone (Foxo1, 3.1; $P < 0.005$, Trim63, 3.2-fold; $P < 0.05$; Fbxo32, 5.2-fold; $P < 0.0005$) (Fig. 5b–d, Additional file 4: Figure S3). These results were supported by a marked increase in protein expression of both phosphorylated and total Foxo1 in WT and TNF-tg animals in response to corticosterone (Fig. 5j). In contrast, expression of anabolic factors such as EF2 did not differ between groups. A deeper analysis of muscle gene expression was performed examining catabolic signalling and E3 ligases (*Foxo1, Fbxo32, Trim63, Ube3a*), anabolic and anti-anabolic myokines and signalling (*Igf1, Igf2, Mstn, Redd1*), muscle differentiation (*Myog, MyoD, Myf5, Myf6*) and inflammatory myokines and signalling (*Tnfa, Il6, Cxcl1, IkBa*) (Additional file 3: Figure S2a-p). In addition to the upregulation of all atrogenes examined, gene expression of the inflammatory myokines *Il6* and *Cxcl1* showed

evidence of suppression in response to corticosterone in line with expected anti-inflammatory action. Similar observations were apparent in primary cultures of murine muscle cells treated with corticosterone. Here, a significant upregulation was observed in Trim63 and Fbxo32 regardless of inflammatory stimulation with TNFα (Fig. 5e–i). These data demonstrate that at therapeutic doses, GCs result in a significant induction in anti-anabolic and catabolic gene expression in muscle.

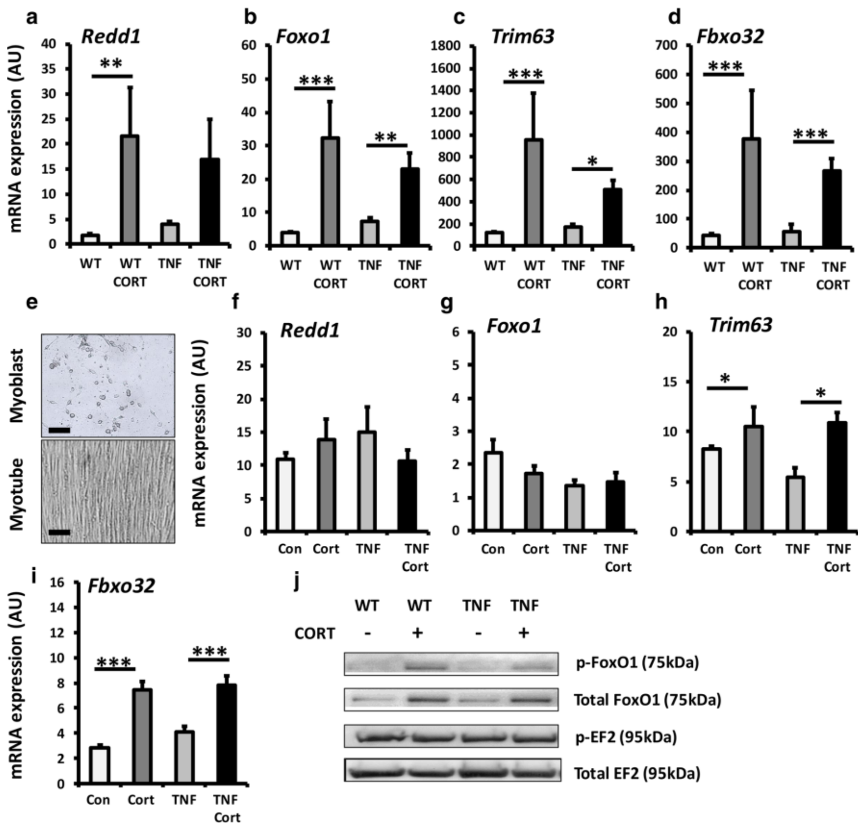

Figure 5. a–d Quantification of gene expression (arbitrary units) of Redd1, Foxo1, Trim63 and Fbxo32, determined by RT qPCR, in ex vivo quadriceps biopsies. **e–h** Representative images of primary murine myoblast and myotubes and quantification of gene expression of Redd1, Foxo1, Trim63 and Fbxo32, determined by RT qPCR and **j** representative western blot staining for p-Foxo1, total Foxo1, pEF2 and p-EF2 after loading of 20 µg of protein and normalisation to ponceau staining in quadriceps for either WT mice and TNF-Tg animals receiving either vehicle or corticosterone (100 µg/mL) in drinking water over 3 weeks or in primary myotubes treated with either vehicle, TNFα (10 ng/ml), corticosterone (1 µmol/l) or a combination of TNF and corticosterone for 48 h. Values are expressed as mean ± standard error of six animals or primary cultures derived from four separate animals. Statistical significance was determined using two-way ANOVA with a Tukey post hoc analysis. *P < 0.05,

5.4 Discussion

We employed a murine model of polyarthritis treated with oral corticosterone to examine the effects of GCs on bone and muscle in the context of initial preventative bridging therapy in early-onset polyarthritis. This approach was effective at suppressing disease activity and joint destruction and has previously been demonstrated to mimic the kinetics of oral GC therapy [22]. Clinically, GCs suppress disease activity, joint destruction and systemic inflammation, which are drivers of bone and muscle loss in RA [1, 23-26]. However, whilst effective in controlling disease activity, they are independently associated with GC-induced osteoporosis and muscle wasting through direct anti-anabolic and catabolic pathways [8-13, 27, 28]. Currently, their impact on bone and muscle when used to treat new-onset inflammatory arthritis remains unclear.

The anti-inflammatory properties of oral GC administration were evidenced by a marked suppression in disease activity, joint inflammation and joint destruction in the TNF-tg model. We observed a marked decrease in trabecular bone volume at day 53 in TNF-tg mice, characterised by an increase in osteoclast activity and numbers. Administration of oral GCs resulted in a significant protection from this inflammatory bone loss.

Increased osteoclast numbers and activity are recognised mediators of inflammatory bone loss in RA, whilst therapeutic control of inflammation abrogates this [29-31]. Our study mirrors these findings, where TRAP +ve osteoclast numbers

and bone resorption determined by serum CTX1 were significantly reduced in animals receiving corticosterone. In vitro data supported these findings where osteoclast numbers and activity were markedly reduced by GCs in TNFα-stimulated osteoclasts. These data indicate that the protection from inflammatory bone loss in TNF-tg animals receiving corticosterone are mediated through the suppression of osteoclastic bone resorption.

Both inflammation and GCs are reported to be negative regulators of bone formation and osteoblastogenesis [32-34]. Here, the anti-anabolic actions of GCs on bone formation are associated with a rapid suppression of P1NP and reduction in trabecular bone in patients [34].

In our study, whilst markers of mature osteoblasts and bone formation were suppressed in TNF-tg animals, therapeutic GCs dramatically exacerbated this and mirror observations in patient studies. These data suggest that the direct anti-anabolic actions of GCs outweigh positive effects of suppressing inflammation on bone formation in vivo.

Several studies have explored the effects of therapeutic GCs on bone metabolism in RA. These yielded conflicting outcomes and are complicated by differences in disease severity, parallel DMARD therapy and dosing of GCs. Several reported increased fracture risk, with complications more apparent at higher doses [23, 35-37]. Others reported no worsening of bone mineral density or fracture risk when given at low doses in combination with DMARDS [3, 38, 39].

In contrast to bone loss, oral GCs increased muscle wasting and did not restore animal mobility in the TNF-tg model of polyarthritis, despite effective suppression of disease activity. This was characterised by a comparable decrease in muscle wet weights and fibre size in WT and TNF-tg animals suggesting that these changes occur independent of inflammatory muscle wasting and that GC-driven muscle wasting impacts on animal mobility.

In patients receiving therapeutic GCs, muscle wasting is characterised by increased protein breakdown driven through the ubiquitin proteasome and the lysosomal systems [11-13, 28]. Our results mirror this with an upregulation of anti-anabolic and catabolic pathway activation in animals receiving corticosterone.

Similar results were observed in primary muscle cultures, where regardless of pro-inflammatory stimulation with TNFα, a direct induction of catabolic gene expression was observed in response to GCs. Additive or synergistic catabolic actions in muscle by oral GCs in combination with inflammation were not observed in this study. This is most likely attributed to the effective suppression of well-defined inflammatory mediators of muscle wasting that are themselves suppressed by GCs [40-42].

Few studies address therapeutic GC use on muscle wasting in RA. However, of note, a recent study by Lemmey et al. reported a rapid loss of muscle mass in RA patients receiving a single intramuscular injection of GCs to treat disease flares [43]. Unfortunately, this study was not able to address the impact of disease activity (itself

a significant driver of muscle wasting) and DMARD use on muscle wasting independent of corticosterone use. However, our study supports the author's conclusions that muscle wasting is an immediate and severe complication in RA patients receiving GCs, independent of inflammation. Indeed, the marked increase in fracture risk upon initiation of GCs in RA may be primarily driven by GC-induced muscle wasting, rather than secondary to GC-induced osteoporosis [44].

5.5 Conclusions

Using an animal model of chronic polyarthritis, this study examined how controlling disease activity with oral GCs as a monotherapy influenced inflammatory osteoporosis and muscle wasting. We demonstrate that when given in early disease, oral GCs protect against inflammatory bone loss, but induce marked systemic muscle wasting. These results suggest that the development of interventions to manage deleterious side effects in muscle should be prioritised in patients with inflammatory arthritis receiving GCs.

Author Contributions

Animal experiments and analysis of in vivo and ex vivo data were performed by FCG, and WJM carried out the experiments with support from MCS, SAP and LYC. In vitro experiments were performed and analysed by FCM, WC, FS and LJW and designed by RSH, GCS and JS. RSH, KR, LR and LGG designed and supervised the project with support from MSC. All authors discussed the results and contributed to the final manuscript. All authors read and approved the final manuscript. Fenton CG and Webster JM shared joint authorship.

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Supplementary data.

Primary culture media	Media	Supplements
Human Osteoblast Culture Media	High glucose Dulbecco's Modified Eagle Medium (DMEM)	10 % fetal calf serum, 1 % sodium pyruvate, 1 % non-essential amino acids, 1 % penicillin-streptomycin, 50 µg/ml ascorbic acid 2 mM β-glycerophosphate
Human Osteoclast Culture Media	α-MEM	10 % fetal calf serum, 0.02 mM L-glutamine, 1 % penicillin-streptomycin, 25 ng/ml M-CSF (PeproTech)
Murine Muscle Expansion Media	High glucose Dulbecco's Modified Eagle Medium (DMEM)	30 % fetal calf serum, 10 % horse serum 1 % Chick Embryo Extract 10 ng/ml basic fibroblast growth factor (PeproTech)
Murine Muscle Maintenance Media	High glucose Dulbecco's Modified Eagle Medium (DMEM)	10 % horse serum, 0.5 % Chick Embryo Extract
Muscle Differentiation Media	High glucose Dulbecco's Modified Eagle Medium (DMEM)	2 % horse serum

Supplementary Table 1: Media for primary cultures

Real-time PCR Master Mix	
2X TaqMan PCR Master mix (Life Technologies)	5 µl
200 nmol TaqMan probe	0.5 µl
RNAse free water	3.5 µl
cDNA	25–50 ng in 1 µl

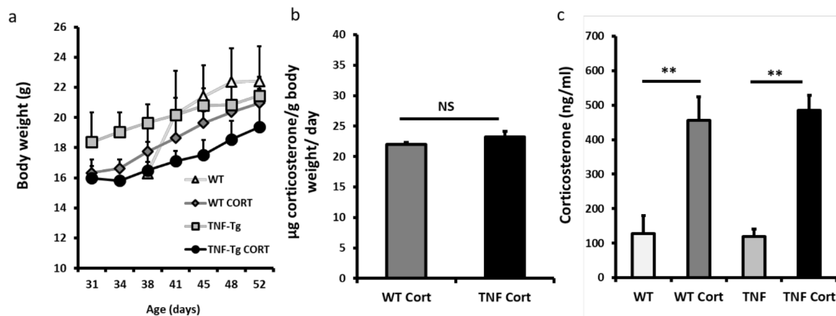
Supplementary Table 2: Real-time PCR Master mix

Buffers	
Sucrose Lysis buffer	50 mM Tris/HCl (pH 7.5), 250 mM Sucrose 1 mM EGTA 50 mM NaF 10 mM Na-β-Glycerophosphate, 1 mM Benazmidine 1 % Triton X-100 0.1 % β-Mercaptoethanol 5 mM Na-Pyrophosphate, 1 mM EDTA 1 mM Na3VO4 supplemented with protease inhibitor cocktail
Blocking buffer, Tris-buffered saline Tween-20	0.137 M NaCl 0.02 M Tris-base 7.5pH 0.1 % Tween-20 5 % skimmed milk in (TBS-T, 0.137M)
TRAP buffer (pH 5)	0.1 M sodium acetate, 75 mM L+ tartaric acid, 1.6 mM Fast Red Violet LB salt 0.5 % naphthol AS-MX phosphate

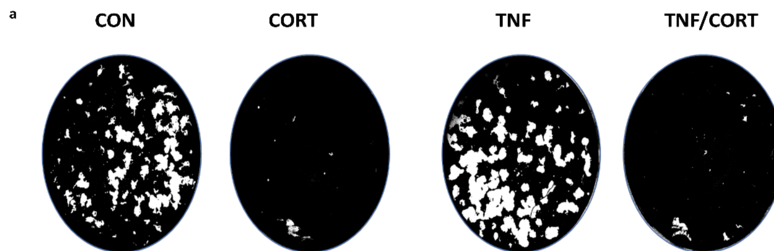
Supplementary Table 3: Buffers

IgG	Manufacturer	Cat No	Dilution
FoxO1/FoxO3a T24/T32	Cell Signaling Technology	#9464	(1/500)
p-FoxO1/FoxO3a	Cell Signaling Technology	#2880	(1/1000)
eEF2	Cell Signaling Technology	#2332	(1/5000)
p-eEF2 T56	Cell Signaling Technology	# 2331	(1/5000)

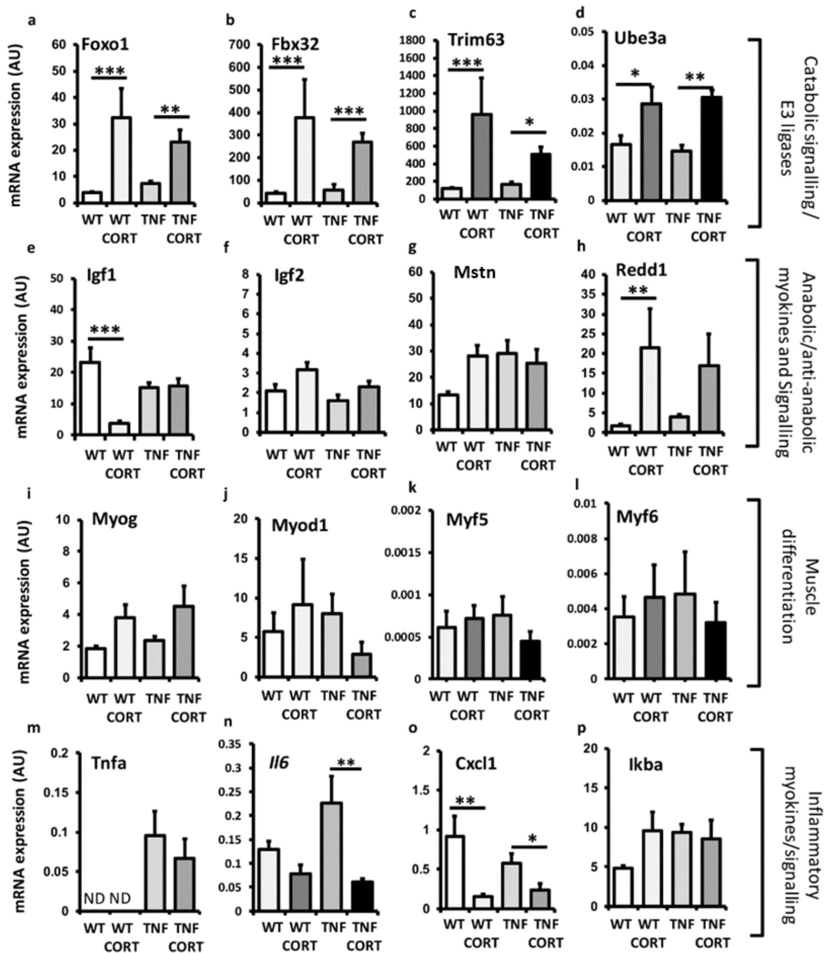
Supplementary Table 4: IgGs used in Immunoblotting



Supplementary Figure 1: (**a**) body weights (g), (**b**) daily corticosterone intake (µg/g body weight/day) and (**c**) serum corticosterone determined by ELISA (ng/ml) in in WT and TNF-Tg animals receiving either vehicle or corticosterone (100 µg/mL) in drinking water over 3 weeks. Values are expressed as mean ± standard error of six per group for weight and at least three animals per group for steroid intake and serum measurement. Statistical significance was determined using two-way ANOVA with a Tukey post hoc analysis. * $P < 0.05$, **$P < 0.005$, ***$P < 0.001$. (TIF 435 kb).



Supplementary Figure 2: (**a**) Representative images of human primary culture osteoclast activity assessed on mineral-coated plates at day 14 treated with vehicle, cortisol (1000 nmol/l), TNFa 10 ng/ml) or a combination of both. Images were acquired using EVOS FL Auto Cell Imaging System (Life Technologies). (TIF 1090 kb).



Supplementary Figure 3: (**a-p**) Gene expression of Foxo1, Fbxo32, Trim63, Ube3a, Igf1, Igf2, Mstn, Redd1, Myog, MyoD, Myf5, Myf6, Tnfa, Il6, Cxcl1 and IkBa were determined by RT-qPCR in quadriceps for either WT mice and TNF-Tg animals receiving either vehicle or corticosterone (100 µg/mL) in drinking water over 3 weeks. Values are expressed as mean ± standard error of six animals or primary cultures derived from four separate animals. Statistical significance was determined using two-way ANOVA with a Tukey post hoc analysis. *P < 0.05, **P < 0.005, ***P < 0.001. (TIF 1454 kb).

Chapter 6 – Global deletion of 11β-HSD1 prevents muscle wasting associated with glucocorticoid therapy in polyarthritis

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Abstract

Glucocorticoids provide indispensable anti-inflammatory therapies. However, metabolic adverse effects including muscle wasting restrict their use. The enzyme 11beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) modulates peripheral glucocorticoid responses through pre-receptor metabolism. This study investigates how 11β-HSD1 influences skeletal muscle responses to glucocorticoid therapy for chronic inflammation. We assessed human skeletal muscle biopsies from patients with rheumatoid arthritis and osteoarthritis for 11β-HSD1 activity ex vivo. Using the TNF-α-transgenic mouse model (TNF-tg) of chronic inflammation, we examined the effects of corticosterone treatment and 11β-HSD1 global knock-out (11βKO) on skeletal muscle, measuring anti-inflammatory gene expression, muscle weights, fiber size distribution, and catabolic pathways. Muscle 11β-HSD1 activity was elevated in patients with rheumatoid arthritis and correlated with inflammation markers. In murine skeletal muscle, glucocorticoid administration suppressed *IL6* expression in TNF-tg mice but not in TNF-tg^{11βKO} mice. TNF-tg mice exhibited reductions in muscle weight and fiber size with glucocorticoid therapy. In contrast, TNF-tg^{11βKO} mice were protected against glucocorticoid-induced muscle atrophy. Glucocorticoid-mediated activation of catabolic mediators (*FoxO1*, *Trim63*) was also diminished in TNF-tg^{11βKO} compared to TNF-tg mice. In summary, 11β-HSD1 knock-out prevents muscle atrophy associated with glucocorticoid therapy in a

model of chronic inflammation. Targeting 11β-HSD1 may offer a strategy to refine the safety of glucocorticoids.

Keywords: Sarcopenia, myopathy, steroids, adverse effects, 11beta hydroxysteroid dehydrogenase 1, rheumatoid arthritis, inflammation

6.1 Introduction

Glucocorticoids are indispensable in modern medicine as highly effective anti-inflammatory medications. They are widely used with applications ranging from autoimmune diseases to cancer, organ transplantation, or COVID-19 pneumonia. Their capacity to control inflammation relies on direct action on immune cells [1, 2]. However, action on tissues besides the immune system leads to a characteristic pattern of adverse effects that restricts their long-term use [2, 3]. In skeletal muscle, glucocorticoids cause weakness and atrophy through pathways including reduced anabolic insulin-like growth factor 1 (IGF1)/insulin signaling and enhanced catabolic signaling via the ubiquitin-proteosome system [4]. Patients suffer detrimental consequences from muscle wasting in the form of reduced mobility, higher falls and fracture risk, higher hospitalization rates and increased mortality [5].

The enzyme 11beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) modulates the signal strength of glucocorticoids within target tissues, including in skeletal muscle [6]. It converts inactive glucocorticoids (cortisone or prednisone in humans; dehydrocorticosterone (DHC) in rodents) to active glucocorticoids (cortisol/prednisolone/corticosterone), amplifying the local glucocorticoid signal. 11β-HSD1 has been implicated in a range of metabolic pathologies [6]. In particular, suppression of 11β-HSD1 protects against muscle wasting from glucocorticoid excess, highlighting 11β-HSD1 as a potential target for therapeutic intervention [7,

8]. Pharmaceutical inhibitors of 11β-HSD1 have been developed and are in clinical trials for diabetes and other metabolic diseases [9-11].

Inflammation enhances the activity of 11β-HSD1. Inflammatory cytokines such as TNF-α upregulate 11β-HSD1 expression, providing a local negative feedback to control inflammation with local anti-inflammatory glucocorticoid activation [12, 13]. Inflammation also acts systemically to augment circulating glucocorticoids (activation of the hypothalamic-pituitary-adrenal (HPA) axis), as well as acting directly on skeletal muscle to cause myopathy [14]. Hence, compound effects on skeletal muscle depend on complex interactions between inflammation, 11β-HSD1 activity and glucocorticoid signaling.

Our previous work showed that both the genetic deletion of 11β-HSD1 and exogenous glucocorticoid, administered at a dose sufficient to suppress disease activity, exacerbate the muscle wasting phenotype in a model of polyarthritis with chronic inflammation [13, 15]]. This study addresses whether suppression of 11β-HSD1 function will protect or harm skeletal muscle metabolism when chronic inflammation and exogenous glucocorticoids are combined in a murine model of glucocorticoid-treated polyarthritis. Our data show that global 11β-HSD1 knock out (11βKO) has a protective effect against skeletal muscle wasting when mice with chronic TNF-α overexpression receive oral corticosterone. This offers new insights into the driving factors for muscle wasting in glucocorticoid-treated

inflammatory disease, with implications for strategies to mitigate the adverse effects of glucocorticoid treatments in the future.

6.2 **Materials and methods**

All biochemical reagents used are from Sigma, Dorset, United Kingdom, unless stated otherwise.

6.2.1 Human Skeletal Muscle Biopsies

Adult patients with hip osteoarthritis (OA) or rheumatoid arthritis (RA) consented to collection of quadriceps muscle biopsies (150–200 mg) during elective joint replacement surgery, following ethical approval (REC reference 14/ES/1044 & NRES 16/SS/0172). Current glucocorticoid therapy was an exclusion criterion. Fresh muscle tissue was used either immediately for enzymatic activity analysis, or snap-frozen in liquid nitrogen for later real-time PCR analysis.

6.2.2 Animal Models

Transgenic mice with chronic systemic overexpression of human *TNF-α* (TNF-tg mice) were used as a model for chronic inflammation and polyarthritis. TNF-tg animals were provided by Professor George Kollias (Biomedical Sciences Research Center 'Alexander Fleming', Athens, Greece). This model, first described by

Keffer et al. [16], relies on replacement of the 3′-untranslated region of the human TNFα gene with the 3′-untranslated region of the β-globin gene, which greatly increases transcriptional efficiency, stability and expression. Human TNFα has homology with murine TNFα and effectively binds murine TNF-R1 receptors, but not murine TNF-R2 receptors, resulting in less severe inflammatory manifestations than chronic overexpression of murine TNFα [17, 18]. Transgene expression affects multiple tissues (joints, brain, kidney, spleen, thymus) with confirmed expression in murine skeletal muscle tissue [13, 16]. We have previously demonstrated high levels of human TNFα transgene expression and equivalent levels of native murine TNFα expression in skeletal muscle of TNF-tg mice compared to wild-type mice [13]. The characteristic phenotype of these animals involves progressive polyarthritis from 6 weeks of age. The inflammatory and musculoskeletal phenotype of these TNF-tg mice has previously been described [13, 15, 19]. TNF-tg mice were crossed with 11β-HSD1 global KO mice (11βKO) as previously described to generate TNF-tg^{11βKO} animals [20]. All animals were maintained on C57BL/6 background and littermates with intact 11β-HSD1 gene served as respective controls. Preserved human TNFα transgene expression in skeletal muscle tissue of double mutant mice has previously been validated [13] and was confirmed for this study (Supplementary Figure S1).

Male mice were housed in standard conditions with ad lib access to standard chow and water. Animals were scored twice weekly for clinical parameters

of inflammation using a validated template [13, 19] from 28 days of age until the end of the experiment. Treatment with glucocorticoids or vehicle started from 32 days of age, coinciding with onset of measurable polyarthritis, and continued for 3 weeks. Drinking water was supplemented with either corticosterone (100 µg/mL, 0.66% ethanol), or vehicle (0.66% ethanol), with an average consumption of 1.25 mg per day. Mice were sacrificed at the end of 3 weeks' treatment. Adrenal glands, tibialis anterior (TA) and quadriceps muscle were dissected, weighed and weights normalized to total body weights. Muscles were snap frozen in liquid nitrogen and stored at −80 °C for later biochemical analysis. Experiments complied with the Animal (Scientific Procedures) Act 1986 and received approval from the Birmingham Ethical Review Subcommittee (project license P51102987).

6.2.3 11β-HSD1 Enzymatic Activity Assay

Confluent cells or fresh tissues were incubated in medium containing 100 nmol/L cortisone (for human samples) or 100 nmol/L DHC (for rodent samples) along with tracer amounts of tritiated cortisone or tritiated DHC (Perkin Elmer, Beaconsfield, UK). Steroids were extracted in dichloromethane and separated by thin-layer chromatography with ethanol/chloroform (8:92) as the mobile phase. Thin-layer chromatography plates were analyzed with a Bioscan imager (Bioscan, Washington, DC, USA), and the fractional conversion of steroids was calculated. The protein concentration was determined with the Bio-Rad Protein assay using the

Bradford method (Bio-Rad, Hercules, CA, USA). Experiments were performed in triplicate, and enzymatic activity is reported as pmol product per mg protein per hour.

6.2.4 Primary Murine Muscle Cell Culture

Murine TA muscles were used to generate primary cultures of differentiated myotubes as previously described [21, 22]. TA muscles were removed from WT and 11βKO mice at 9 weeks and digested in type 1 collagenase at 37 °C for 2 h, before isolating individual fibers. Fibers were plated in satellite media (DMEM High Glucose, 30% FBS, 10% HS, 1% Chick Embryo Extract, 10 ng/mL basic fibroblast growth factor) and grown in Matrigel™-coated plates (Corning Life Sciences, Flintshire, UK). Satellite cells migrating from muscle fibers were removed and cultured in proliferation medium (DMEM High Glucose, 10% HS, 0.5% Chick Embryo Extract) until confluent. Primary myoblasts were then grown in differentiation medium (DMEM High Glucose, 2% HS) for five days until syncytialized myotubes formed. To investigate effects of inflammation and glucocorticoids, differentiated myotubes were incubated in media with added TNF-α (10 ng/mL) and/or dehydrocorticosterone (1 µmol/l) for 24 h.

6.2.5 *RNA Isolation and Analysis of Gene Expression*

RNA was extracted by mechanical suspension and lysis of muscle in TRIzol™ reagent (Thermo Fisher Scientific, Loughborough, UK). Phase separation and RNA precipitation was performed with the addition of chloroform and 2-propanol. RNA precipitates were reconstituted in RNase-free water and stored at −80 °C. cDNA was generated by reverse transcription in accordance with the manufacturer's protocol (Multiscribe™, Thermo Fisher Scientific, Loughborough, UK). Expression of specific genes was assessed by real-time PCR using TaqMan® Gene Expression Assays (Thermo Fisher Scientific, Loughborough, UK) on an ABI7500 system (Applied Biosystems, Warrington, UK). Final reactions 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 normalized to the housekeeper gene GAPDH by calculating ΔCt values (Ct target − Ct GAPDH). For graphical illustrations, standardized expression values were transformed with the formula $(2^{-\Delta Ct}) \times 1000$. For describing changes between experimental and control groups, the difference of ΔCt values as calculated as ΔΔCt = ΔCt[experimental group] − ΔCt[control group] and reported as fold change = $2^{\Delta\Delta Ct}$.

6.2.6 *Histological Analysis of Muscle*

Murine quadriceps muscles were embedded in paraffin and cut to 10 µm sections for histology. Samples were stained with hematoxylin and eosin prior to

quantitative analysis of fiber size distribution using Image J software [23]. Measurements were taken in three 200 μm^2 regions of the vastus medialis for six mice per group.

6.2.7 Western Blots

Protein content of quadriceps muscles was homogenized in an ice-cold lysis buffer (50 mM Tris, 1 mM EDTA, 10 mM Na-B-Glycerophosphate, 5 mM Na-Pyrophosphate, 1 mM Benzamidine, 250 mM Sucrose, 50 mM NaF, 0.1% β-Mercaptoethanol, 1 mM Na2VO3, 1% Triton X and Protease Inhibitor Cocktail (Merck Life Science, Dorset, UK)). Proteins were denatured in Laemmli buffer at 95 °C for 5 min. 20 μg of proteins were resolved in Tris-glycine SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. After blocking, membranes were probed with the primary antibody overnight at 4 °C. All primary antibodies were obtained from Cell Signaling Technology, Inc. and diluted at 1:1000 in TBS-Tween plus 5% BSA or skimmed milk (p-FOXO1 #9464, FOXO1 #2880, p-S6 Ribosomal Protein (Ser235/236) #4856, S6 Ribosomal Protein #2217). Signal detection used horseradish peroxidase-conjugated secondary antibodies and ECL substrate. The membrane was stained with Ponceau S solution (0.2% Ponceau S in 1% acetic acid; Sigma-Aldrich Chemie) to control for protein loading.

6.2.8 *Statistical Analysis*

Data was analyzed by unpaired *t*-test, one way and two-way ANOVA with Tukey post-hoc analysis or Pearson correlation analysis, or non-parametric equivalent tests as appropriate using GraphPad Prism [24]. Aggregate data are reported as mean ± standard error, unless otherwise specified. Statistical significance was defined as p-value < 0.05 (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; no asterisk or NS $p > 0.05$).

6.3 Results

6.3.1 *Cortisol Activation by 11β-HSD1 Is Increased in Skeletal Muscle in Rheumatoid Arthritis and Correlates with Inflammation*

Inflammatory upregulation of 11β-HSD1 has previously been demonstrated when skeletal muscle samples from patients with osteoarthritis (OA) were stimulated with TNF-α ex vivo [13]. Here, we investigated whether inflammatory upregulation of 11β-HSD1 is evident in muscle biopsies from patients with rheumatoid arthritis (RA), a condition with chronic systemic inflammation. Patients with OA and RA had similar age, but systemic inflammation markers were higher in patients with RA including C-reactive protein (CRP) and erythrocyte sediment rate (ESR) (Table 1). Exclusion criteria stipulated that no patient received current oral glucocorticoid therapy. Measuring ex vivo cortisone to cortisol conversion rate, we confirmed that 11β-HSD1 enzymatic activity was higher in muscle biopsies taken

from patients with RA compared to patients with OA (mean ± std. error: 0.014 ± 0.001 pmol/mg tissue/h vs. 0.010 ± 0.001 pmol/mg tissue/h respectively, $p < 0.05$; Figure 1A). Among patients with RA, muscle 11β-HSD1 activity correlated with circulating CRP levels ($R^2 = 0.131$, $p = 0.05$; Figure 1B). Real-time PCR showed higher expression of the inflammatory cytokine *IL6* in muscle samples from patients with RA compared to patients with OA (fold change = 8.65, $p < 0.05$; Figure 1C). Expression of this tissue-specific marker of inflammation correlated with expression of 11β-HSD1 among patients with RA ($R^2 = 0.369$, $p < 0.05$; Figure 1D). Similarly, expression of the inducible inflammatory activator *COX2* correlated with 11β-HSD1 expression in muscle biopsies from patients with RA ($R^2 = 0.672$, $p < 0.05$; Figure 1E). Finally, muscle samples from patients with RA compared to OA exhibited higher expression of Forkhead box protein O1 (*FOXO1*) and myostatin (*MSTN*), negative regulators of muscle growth (fold changes 2.35, $p < 0.05$ and 3.62, $p < 0.05$ respectively; Figure 1F,G). Taken together, these data demonstrate that 11β-HSD1 activity is elevated in the skeletal muscle of patients with RA relative to patients with OA, and that 11β-HSD1 upregulation correlates with systemic and tissue-specific markers of inflammation.

Patient details	Patients with OA (n=12)	Patients with RA (n=10)	Group comparison (p-value)
Age (years)	66.2 + 3.3	65.7 + 4.1	0.93
CRP (mg/L)	2.3 + 1.7	11.0 + 3.5	0.03
ESR (mm/h)	1.9 + 1.0	24.13 + 7.1	0.001
Methotrexate (n)	0	5	na

Table 1: Patients' characteristics for human skeletal muscle biopsies.

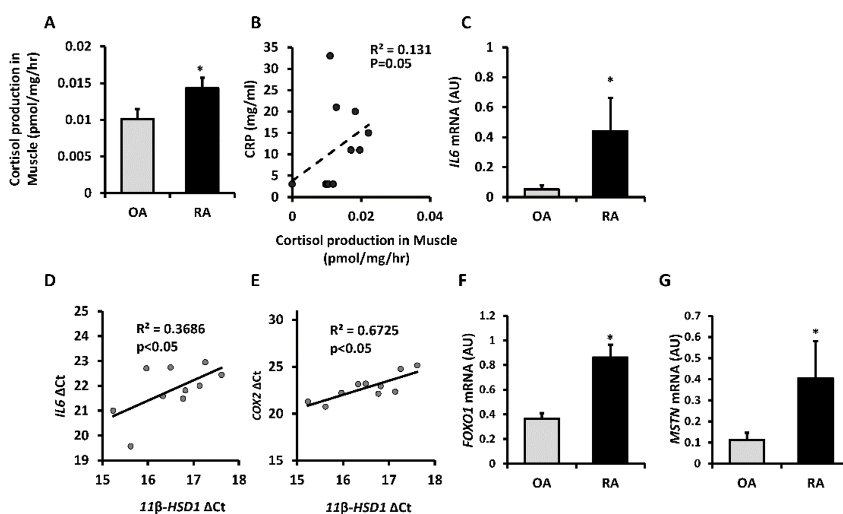

Figure 1. (**A**) Glucocorticoid activation by 11β-HSD1 in ex vivo muscle explants freshly isolated after joint replacement surgery from patients with rheumatoid arthritis (RA, n = 10) and osteoarthritis (OA n = 12) determined by scanning thin-layer chromatography. (**B**) A significant correlation was seen between glucocorticoid activation (oxoreductase activity) and the serum C-reactive protein (CRP) measured before surgery in RA patients. (**C**) Gene expression (AU) of IL6 determined by RT qPCR in muscle homogenates from RA (n = 6) and OA (n = 4) patients. (**D**) Correlation between gene expression (ΔCt) of 11β-HSD1 with IL6 and (**E**) COX2 determined by RT qPCR in muscle homogenates from RA (n = 6) and OA (n = 4) patients. (**F**) Gene expression (AU) of FOXO1 and (**G**) MSTN determined by RT qPCR in muscle homogenates from RA (n = 5) and OA (n = 4) patients. Values are expressed as mean ± standard error. Statistical significance was determined using an unpaired t-test. * $p < 0.05$.

6.3.2 *Deletion of 11β-HSD1 in a Model of Chronic Inflammation Causes Resistance to the Anti-Inflammatory Responses of Therapeutic Glucocorticoid in Muscle*

11β-HSD1 function has emerged as essential for glucocorticoid therapy to suppress joint inflammation in models of polyarthritis [20]. We therefore investigated how deletion of 11β-HSD1 affects anti-inflammatory responses in muscles when mice with inflammation from transgenic human TNF-α overexpression are treated with oral corticosterone. The skeletal muscle tissue from 11βKO mice exhibited no detectable activity to activate DHC to corticosterone (Figure 2A). Systemic treatment with corticosterone led to a 50% adrenal weight reduction in TNF-tg mice relative to untreated controls ($p < 0.05$; Figure 2B). For TNF-tg^{11βKO} mice, adrenal weight reduction with corticosterone treatment was 60% relative to untreated counterparts ($p < 0.05$; Figure 2B). Oral corticosterone treatment in TNF-tg mice led to a trend for higher muscle mRNA expression of *Gilz*, a glucocorticoid responsive gene with anti-inflammatory properties (Figure 2C). However, such a trend for corticosterone-induced *Gilz* expression was not apparent in TNF-tg^{11βKO} mice. In direct comparison of corticosterone-treated groups, muscle *Gilz* expression was significantly higher in TNF-tg mice than in TNF-tg^{11βKO} mice (fold change = 2.98, $p < 0.05$). Examination of *Il6* mRNA expression in muscle yielded consistent results. While corticosterone administration suppressed expression of pro-inflammatory *Il6* in TNF-tg mice (fold change = 0.27, $p < 0.05$; Figure 2D), corticosterone was rendered ineffective in TNF-tg^{11βKO} mice (fold

change = 0.96, *p*-value NS). Primary muscle cultures of WT and 11βKO animals confirmed that 11βKO muscle cells are unresponsive to the inactive glucocorticoid DHC and its immunosuppressive effects. DHC does not activate the glucocorticoid receptor directly but relies on conversion to active corticosterone by 11β-HSD1 to trigger glucocorticoid responses. Accordingly, DHC stimulation markedly enhances *Gilz* mRNA expression in WT muscle cells, but not in 11βKO cells (Figure 2E,F). Similarly, DHC stimulation markedly suppresses TNF-induced *Il6* mRNA expression in WT muscle cells, but not in 11βKO cells (Figure 2G,H). Together, these data reveal that anti-inflammatory responses of glucocorticoids in skeletal muscle are dependent on 11β-HSD1.

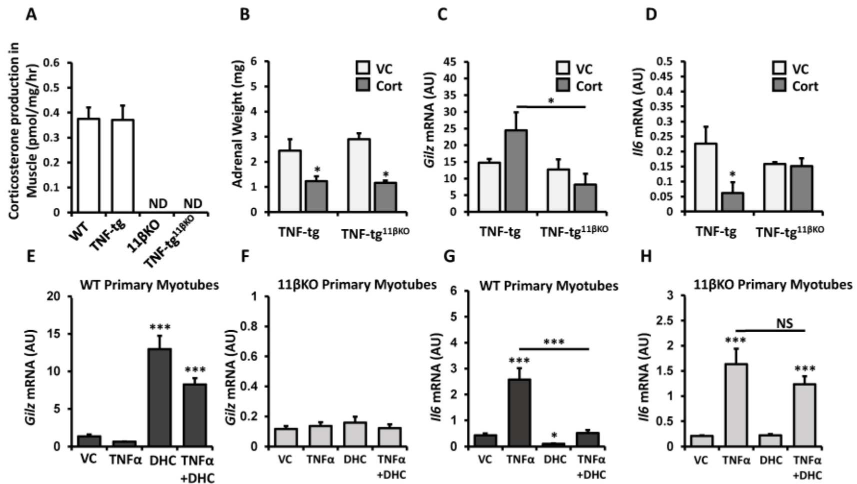

Figure 2. (**A**) Corticosterone production (pmol/mg/hour) determined by scanning thin layer chromatography in ex vivo tibialis anterior muscle biopsy isolated from wild type (WT), TNF-transgenic (TNF-tg), 11β-HSD1 knock out (11βKO) and $\text{TNF-tg}^{11\beta KO}$ animals. (**B**) Adrenal weights and gene expression (AU) of (**C**) Gilz and (**D**) Il6 determined by RT qPCR in muscle homogenates from TNF-tg and $\text{TNF-tg}^{11\beta KO}$ animals receiving either vehicle or corticosterone (100 µg/mL) in the drinking water for 3 weeks. Gene expression (AU) of (**E**,**F**) Gilz and (**G**,**H**) Il6 in primary murine muscle cultures from WT or single mutant 11βKO animals treated with recombinant TNFα and/or dehydrocorticosterone (DHC) determined by RT qPCR. Values are expressed as mean ± SE, n = 6 per group for animal experiments and n = 3 per group for primary culture. Statistical significance was determined using two-way analysis of variance with Tukey post hoc analysis. * $p < 0.05$, *** $p < 0.001$. NS, not significant; WT, Wild type; 11βKO, 11β-HSD1 genetic deletion; VC, Vehicle Control; Cort, Corticosterone; DHC, Dehydrocorticosterone; ND, not detectable.

6.3.3 11β-HSD1 Mediates Muscle Wasting in Response to Therapeutic Glucocorticoids in Chronic Inflammation

Global deletion of 11β-HSD1 has previously been shown to exacerbate myopathy in the inflammatory model of TNF-tg mice but protect against myopathy

in the glucocorticoid excess model of WT mice receiving oral corticosterone [13, 15]. Next, we explored the role of 11β-HSD1 for muscle atrophy in a combined model of TNF-tg mice treated with corticosterone.

Tibialis anterior and quadriceps muscle weights (standardized to total body weights) were assessed in the four experimental groups of vehicle-treated TNF-tg mice, corticosterone-treated TNF-tg mice, vehicle-treated TNF-tg^{11βKO} mice and corticosterone-treated TNF-tg^{11βKO} mice (Figure 3A,B). In TNF-tg mice, corticosterone treatment reduced standardized tibialis anterior and quadriceps muscle weights compared to vehicle control (−33%, $p < 0.05$ and −29%, $p < 0.05$ respectively). In TNF-tg^{11βKO} mice however, corticosterone treatment had no significant effect on muscle weights relative to vehicle treatment (TA: −4%, *p*-value NS and quadriceps: +12%, *p*-value NS). In direct comparison of corticosterone-treated TNF-tg animals and corticosterone-treated TNF-tg^{11βKO} animals, standardized muscle weights were significantly greater in the latter group (TA: +49%, $p < 0.05$ and quadriceps: +43%, $p < 0.01$).

Quantitative analysis of average muscle fiber size and distribution yielded matching results. The average fiber cross-sectional area was significantly larger in corticosterone-treated TNF-tg^{11βKO} mice than in treated TNF-tg mice (57.5 μm^2 vs. 39.0 μm^2, $p < 0.001$; Figure 3C and Supplementary Figure S2). Examined in closer detail, corticosterone treatment for TNF-tg mice produced a left shift in fiber size distribution (i.e., higher frequency of small fibers and lower frequency of large fibers

(Figure 3E)). In contrast, corticosterone treatment for TNF-tg$^{11\beta KO}$ mice produced a right shift in fiber size distribution (Figure 3F). When muscle histology of corticosterone-treated TNF-tg and TNF-tg$^{11\beta KO}$ animals is compared directly, lack of 11β-HSD1 function produces a marked right shift in fiber size distribution (i.e., an abundance of larger fibers and scarcity of smaller fibers (Figure 3G)). To summarize the data from muscle weights and histology, suppression of 11β-HSD1 activity eliminates the atrophic effects of glucocorticoid therapy on skeletal muscle in the context of chronic inflammation.

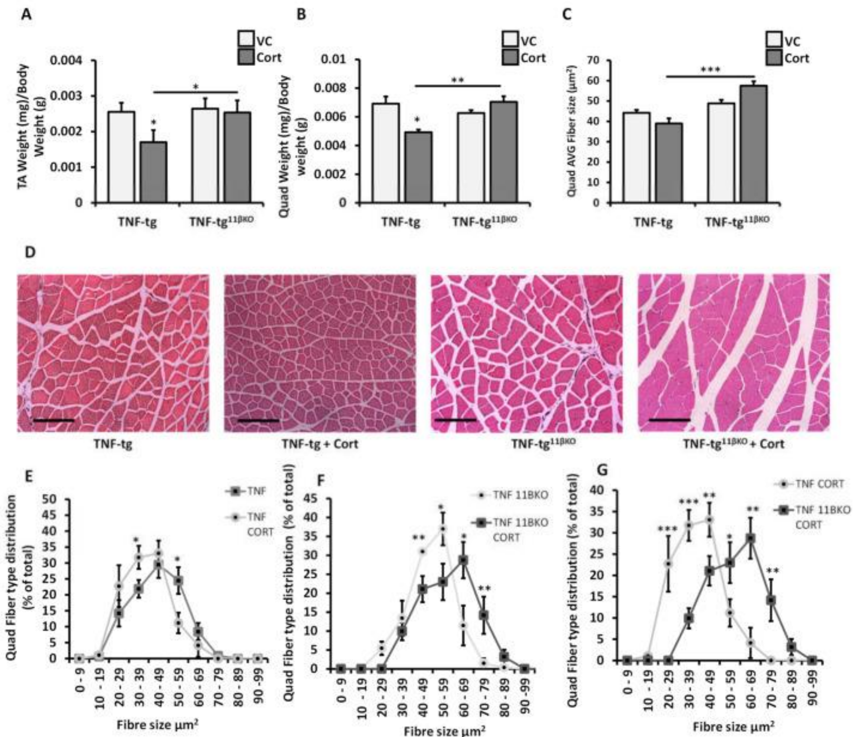

Figure 3. Total (**A**) Tibialis anterior (TA) and (**B**) quadriceps muscle weights relative to total bodyweight and (**C**) average quadriceps muscle fibre cross sectional area (µm2) in TNF-tg, and TNF-tg11βKO animals receiving either vehicle or corticosterone (100 µg/mL) in the drinking water for 3 weeks. (**D**) Representative images of quadriceps muscle sections and (**E–G**) distribution of quadriceps muscle fibre cross-sectional area determined using Image J in paraffin embedded sections in TNF-tg, and TNF-tg11βKO animals receiving either vehicle or corticosterone (100 µg/mL) in the drinking water for three weeks. Values are expressed as mean ± standard error of six animals per group. Statistical significance was determined using two-way ANOVA with a Tukey post hoc analysis. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ (scale bars, 50 µm). VC = Vehicle Control, Cort = Corticosterone.

6.3.4 11β-HSD1 KO Diminishes Activation of Muscle Catabolic Pathways in Response to Therapeutic Glucocorticoid

Inflammation and glucocorticoids are known to mediate muscle atrophy through induction of overlapping catabolic signaling pathways. To elucidate the mechanism by which 11β-HSD1 contributes to muscle wasting in our model of glucocorticoid therapy for chronic inflammation, we examined several regulators of muscle protein metabolism. *FoxO1* is a transcription factor and negative regulator of muscle growth [4]. Corticosterone induced *FoxO1* mRNA expression in muscle of TNF-tg mice (fold change = 3.16, $p < 0.05$), but this response was diminished in animals with 11β-HSD1 deletion (fold change = 2.28, *p*-value NS; Figure 4A). Data on phosphorylation of FOXO1, an inactivating modification, mirrors data on total FOXO1 expression. Corticosterone lowered relative phosphorylation/inactivation of FOXO1 in TNF-tg mice ($p < 0.001$), but not in TNF-tg^{11βKO} mice (*p*-value NS; Figure 4D). *Trim63* (also known as *MuRF1*), an E3 ubiquitin ligase involved in proteolysis, was also upregulated by corticosterone treatment in muscle of TNF-tg mice (fold change = 3.03, $p < 0.01$), but not in TNF-tg^{11βKO} mice (fold change = 1.10, *p*-value NS; Figure 4B). However, the same pattern was less clear for *Fbxo32*, a constituent of the catabolic ubiquitin protein ligase complex. mRNA expression of *Fbxo32* in muscle was upregulated by corticosterone in both TNF-tg and TNF-tg^{11βKO} mice (fold changes 4.75, $p < 0.01$ and 3.24, $p < 0.01$ respectively; Figure 4C). Finally, phosphorylation of ribosomal protein S6, a regulator of mRNA translation and

protein synthesis, was not significantly different between TNF-tg and TNF-tg$^{11\beta KO}$ mice and appeared unaffected by corticosterone treatment in either animal group (Figure 4E,F). To gain further insights, we conducted in vitro experiments using primary muscle cell cultures from WT and 11βKO mice. Expression of myostatin (*Mstn*), an inhibitory factor for muscle growth, increased on stimulation with the inactive glucocorticoid DHC in wild type muscle cells, but not in 11βKO muscle cells (Figure 4G,H). TNF-α did not induce *Mstn*. Expression of *Fbxo32* increased with both DHC and TNF-α stimulation with additive effects in wild type muscle cells, but the response to DHC was again absent in 11βKO muscle cells (Figure 4I,J). Summing up the observations on regulators of muscle metabolism, glucocorticoid therapy in our model of chronic inflammation potently activates muscle catabolic signaling pathways. Genetic deletion of 11β-HSD1 diminishes the glucocorticoid-triggered activation of catabolic signals.

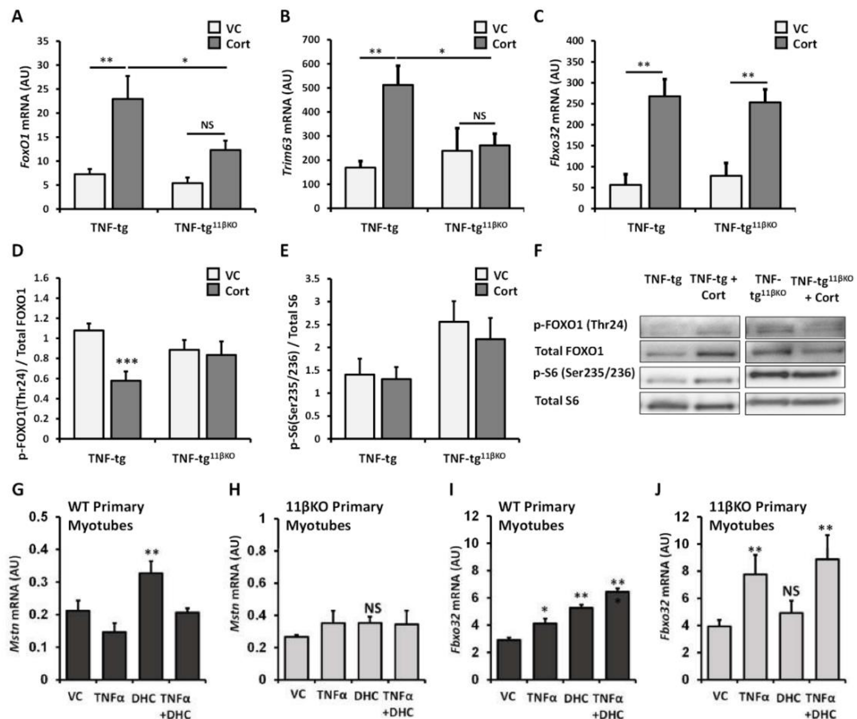

Figure 4. Gene expression (AU) of (**A**) *Foxo1*, (**B**) *Trim63*, and (**C**) *Fbxo32* determined by RT qPCR in muscle homogenates from TNF-tg, and TNF-tg^{11βKO} animals receiving either vehicle or corticosterone (100 µg/mL) in the drinking water for 3 weeks. (**D**) The p-FOXO1(Thr24)/Total FOXO1 and (**E**) p-S6(Ser235/236)/Total S6 ratios and (**F**) representative western blot staining after loading of 20 µg of protein and normalization to Ponceau staining in quadriceps for TNF-tg and TNF-tg^{11βKO} animals receiving either vehicle or corticosterone (100 µg/mL) in the drinking water for 3 weeks. Gene expression (AU) of (**G**,**H**) *Mstn* and (**I**,**J**) *Fbxo32* in primary murine muscle cultures from WT and single mutant 11βKO animals treated with recombinant TNFα and/or dehydrocorticosterone (DHC) determined by RT qPCR. Values are expressed as mean ± SE, n = 6 per group for animal experiments and n = 3 per group for primary culture. Statistical significance was determined using two-way analysis of variance with Tukey post hoc analysis. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. WT, Wild type; 11βKO, 11β-HSD1 genetic deletion; VC, Vehicle Control; Cort, Corticosterone; DHC, Dehydrocorticosterone.

6.4 Discussion

This study reports new knowledge on enhanced glucocorticoid activation in skeletal muscle of patients with rheumatoid arthritis. Furthermore, it provides new insights into the role of 11β-HSD1 for exogenous glucocorticoid therapy in an inflammatory disease model, building on previous research that examined the role of 11β-HSD1 either for exogenous glucocorticoid excess in non-inflammatory models or endogenous glucocorticoid effects in inflammatory models [8, 13]. In the therapeutic model of TNF-transgenic mice receiving oral corticosterone as anti-inflammatory treatment, genetic deletion of the glucocorticoid activating enzyme 11β-HSD1 abolished immunosuppressive responses in muscle. At the same time, 11β-HSD1 deletion mitigated the adverse effects of glucocorticoid therapy on skeletal muscle, protecting against muscle atrophy and diminishing activation of catabolic signaling pathways.

Examination of human skeletal muscle biopsies corroborates that inflammation upregulates enzymatic activity of 11β-HSD1 to activate glucocorticoids locally within muscle. 11β-HSD1 activity was higher for patients with chronic inflammation from rheumatoid arthritis compared to age-matched patients with osteoarthritis. Furthermore, 11β-HSD1 activity correlated with systemic and tissue-specific markers of inflammation among patients with rheumatoid arthritis. The confirmation of 11β-HSD1 upregulation in skeletal muscle of humans with inflammatory disease concords with previous findings. 11β-HSD1 activity in human

muscle rises following ex vivo stimulation with TNF-α [13], or following a major inflammatory insult like abdominal surgery [25]. Furthermore, 11β-HSD1 activity by systemic measures or in tissues besides muscle correlates consistently with inflammation in a range of human observational studies [26-29]. Cell culture experiments clarified that inflammatory cytokines like TNF-α or IL1β directly enhance 11β-HSD1 expression via the NFκB signaling pathway [12, 13]. Altogether, there is strong evidence for upregulation of 11β-HSD1 activity in skeletal muscle by inflammation.

The inflammatory upregulation of 11β-HSD1 in skeletal muscle may provide a physiological defense against excessive inflammatory injury, locally amplifying and targeting immunosuppressive actions of endogenous steroids. Deletion of 11β-HSD1 accordingly exacerbates myopathy in TNF-tg mice with untreated inflammation [13]. Nevertheless, tissue-specific markers of inflammation as well as muscle atrophy markers remained elevated in our cohort with rheumatoid arthritis compared to osteoarthritis. This was despite elevated 11β-HSD1 activity in muscle and in the absence of concurrent glucocorticoid therapy. It remains unclear whether catabolic signaling in this setting of human disease is predominantly due to persisting inflammatory injury or due to augmented atrophic action of endogenous steroids. Future studies with detailed phenotyping following anti-inflammatory or 11β-HSD1 blocking interventions would be needed to address this question.

The significance of 11β-HSD1 function for the anti-inflammatory efficacy of therapeutic glucocorticoids is demonstrated in the TNF-tg animal model. Deletion of 11β-HSD1 led to loss of immunosuppressive responses in skeletal muscle that are normally seen with corticosterone treatment. Morgan et al. previously showed that 11β-HSD1 function is required for glucocorticoid action on muscle in terms of myopathic effects in a model of exogenous glucocorticoid excess [8]. Our data confirm that 11β-HSD1 function is equally important for glucocorticoid action on muscle in terms of immunosuppressive effects in a model of therapeutic glucocorticoid use. These observations in muscle tissue mirror findings from other tissue types. Corticosterone treatment failed to suppress joint inflammation in mouse models of polyarthritis when 11β-HSD1 function was absent [20]. Notably, this is true for administration of active glucocorticoids. Corticosterone treatment led to equivalent adrenal atrophy in TNF-tg and TNG-tg^{11βKO} animals, indicating that glucocorticoid-mediated suppression of the HPA axis was preserved. Considering these findings together suggests that 11β-HSD1 is critical for sustaining or targeting activity of administered glucocorticoids to exert anti-inflammatory effects.

Genetic deletion of 11β-HSD1 protected against muscle wasting in our model of glucocorticoid therapy for inflammatory disease. Results from muscle weights and fiber size distribution were supported by analysis of catabolic signaling pathways. 11βKO diminished activation of *FoxO1* and *Trim63* (also known as *MuRF1*) in muscle of TNF-tg mice receiving corticosterone. *Fbxo32* was not

suppressed in this specific context, suggesting active glucocorticoid administration without signal augmentation by 11β-HSD1 was sufficient for this response. The greater glucocorticoid sensitivity of *Fbxo32* may reflect synergistic activation with TNF, as observed in our in vitro data, possibly via the transcription factor C/EBPβ [30, 31].

Glucocorticoid-induced muscle atrophy was prevented despite failure of immunosuppression and higher inflammatory markers in muscle from 11β-HSD1-deficient TNF-tg^{11βKO} animals relative to the 11β-HSD1-expressing TNF-tg control group. These results are different from observations from untreated TNF-tg mice. 11βHSD1 deletion had no effect on muscle phenotype of vehicle-treated TNF-tg mice in this study, or exacerbated muscle wasting in previous reports [13]. Apparent discrepancies in this regard may be attributed to the timepoint of muscle phenotype assessment, which was earlier in the current study to pre-empt treatment-resistant phenotype divergence. Matching our results from glucocorticoid-treated TNF-tg mice, 11β-HSD1 deletion also mitigated myopathy in wild-type animals receiving excess exogenous glucocorticoid [8]. Altogether, this supports an interpretation that myopathic effects from glucocorticoid therapy predominated over myopathic effects of inflammation in our model of TNF-tg mice treated with oral corticosterone. Further validation of this concept in human disease is needed. Nevertheless, it matches clinical experiences of muscle wasting as a common adverse effect with glucocorticoid therapy for inflammatory disease [3, 32]. In parallel with animal

models receiving exogenous glucocorticoids, glucocorticoid doses for immunosuppression in clinical practice are much above physiological equivalents. The principal observations from our study (elevated 11β-HSD1 activity in muscle in the inflammatory disease rheumatoid arthritis and dependency of adverse glucocorticoid effects on 11β-HSD1 function in the animal model) considered together allow the conclusion that inflammatory upregulation of 11β-HSD1 in skeletal muscle may increase patients' susceptibility to glucocorticoid-mediated myopathy. Therefore, there is a strong argument that 11β-HSD1 activity has a critical contribution to myopathy from glucocorticoid therapy.

Our study highlights a central role of 11β-HSD1 activity for action of glucocorticoids on skeletal muscle, in terms of beneficial anti-inflammatory effects as well as harmful myopathic effects. Using primary muscle cell cultures from wild-type and 11βKO mice without transgenic TNFα expression, we showed that skeletal muscle tissue has sufficient capacity for glucocorticoid activation to downregulate cytokines and upregulate catabolic mediators in an auto-/paracrine manner. Even though minor variations between separate primary cultures are possible, this approach allowed most reliable 11β-HSD1 suppression and a clear delineation of response patterns. Inactive DHC, which requires conversion to corticosterone to trigger glucocorticoid responses, induced GILZ, myostatin and Fbxo32 expression and suppressed TNFα-stimulated IL6 expression in 11β-HSD1 expressing myotubes, but not in myotubes with genetic deletion of 11β-HSD1. Reduced DHC responses for

myostatin and arguably GILZ in the presence of TNF could indicate possible glucocorticoid resistance, a finding that would require further characterization [33].

There remains uncertainty to what extent the observed muscle phenotype in vivo is caused by 11β-HSD1-mediated glucocorticoid activation directly within muscles, as opposed to 11β-HSD1 activity elsewhere in the body. Previous studies suggest that 11β-HSD1 activity in myeloid cells is important for immunosuppression by glucocorticoids in vivo [20], while 11β-HSD1 activity in liver appeared irrelevant for myopathic effects [8]. Variable activity of 11β-HSD1 in different tissue types may determine the balance of beneficial immunosuppressive versus harmful metabolic effects with glucocorticoid therapy. Further research with tissue-targeted glucocorticoid delivery or 11β-HSD1 suppression is needed to clarify this concept and advance the safety of glucocorticoid-based therapies.

Clinical data on the potential benefits of 11β-HSD1 inhibition on muscle metabolism is emerging. Observational studies have reported a correlation of 11β-HSD1 expression in muscle with total lean mass and muscle strength in healthy elderly [34, 35]. Furthermore, a clinical trial found that the 11β-HSD1 inhibitor AZD4017 increased total lean mass in young overweight women [36]. Finally, the TICSI trial is of particular interest in relation to the findings reported here (ClinicalTrials.gov (accessed on 12 July 2021) ID: NCT03111810). It aims to determine whether co-administration of an 11β-HSD1 inhibitor can limit the metabolic side effects of prednisolone, with results expected to report in the near future. However,

safety data will be needed to confirm that 11β-HSD1 inhibitors do not exacerbate inflammatory pathology before using them in patients requiring immunosuppressive therapy. Given that 11β-HSD1 mediates both immunosuppressive and metabolic effects of glucocorticoids, tissue-targeted strategies may hold greater promise than systemic 11β-HSD1 inhibition.

Author contributions

Conceptualization, R.S.H., R.L., M.S.C., K.R. and G.G.L.; investigation, J.M.W., C.G.F. with support from M.S.S., A.P.S., Y.-C.L., S.W.J., A.F. and R.S.H.; writing—original draft preparation, M.S.S.; writing—review and editing, J.M.W., M.S.S., M.S.C., K.R., R.L. and R.S.H.; supervision, R.S.H., R.L., M.S.C., K.R. and G.G.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

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Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki. Research involving human participants was approved by the East of Scotland Research Ethics Service REC 2 (reference 14/ES/1044, date of approval 12 August 2014) and by the South East Scotland Research Ethics Committee 1 (reference 16/SS/0172, date of approval 31 October 2016). Research involving animals complied with the Animal (Scientific Procedures) Act 1986 and received approval from the Birmingham Ethical Review Subcommittee (project license P51102987).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

Data and materials within this manuscript can be made available upon reasonable request to the corresponding author.

Abbreviations

11β-HSD1	11beta-hydroxysteroid dehydrogenase type 1
DHC	dehydrocorticosterone
HPA	hypothalamic-pituitary-adrenal
OA	osteoarthritis
RA	rheumatoid arthritis
TNF-tg	transgenic expression of human TNF-α
11βKO	global genetic deletion of 11β-HSD1
WT	wild-type genotype without genetic modifications
TA	tibialis anterior
NS	not significant
ND	not detectable
ESR	erythrocyte sedimentation rate
CRP	C-reactive protein

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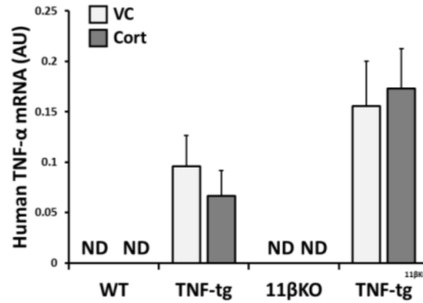
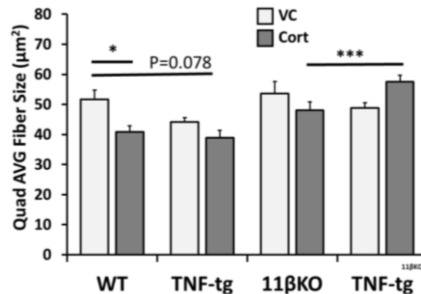
Supplementary Data



Supplementary Figure 1. Gene expression (AU) of human TNFα transgene expression determined by RT PCR in muscle homogenates from wild-type (WT), human TNFα transgenic (TNF-tg), 11β-HSD1 global knock-out (11βKO) and double mutant TNF-tg$^{11\beta KO}$ animals receiving either vehicle or corticosterone (100µg/mL) in the drinking water for 3 weeks. Values are expressed as a mean±SE, n=6 per group. No statistical significant differences were detected for TNF-tg and TNF-tg$^{11\beta KO}$ groups using two-way analysis of variance with Tukey post hoc analysis. WT = Wild type, 11βKO = 11β-HSD1 genetic deletion, VC = Vehicle Control, Cort = Corticosterone, ND = Non-detectable.



Supplementary Figure 2. Average quadriceps muscle fibre cross sectional area (µm^2) in wild-type (WT), human TNFα transgenic (TNF-tg), 11β-HSD1 global knock-out (11βKO) and double mutant TNF-tg$^{11\beta KO}$ animals receiving either vehicle or corticosterone (100µg/mL) in the drinking water for 3 weeks. Values are expressed as a mean±SE, n=6 per group. WT = Wild type, 11βKO = 11β-HSD1 genetic deletion, VC = Vehicle Control, Cort = Corticosterone, Cort = Corticosterone.

Chapter 7 – Summary and General Discussion

The clinical impact of skeletal muscle mass loss in patients with chronic inflammatory disease (CID) is significant, with patients having reduced quality of life and a poor prognostic outcome. Glucocorticoids are a class of steroid hormone that play a central role in regulating energy metabolism, and possess potent immune suppressive, anti-inflammatory properties, resulting in their widespread therapeutic application [1-3]. However, both elevated endogenous GCs and the long-term application of therapeutic GCs result in severe off-target side effects including osteoporosis and systemic muscle wasting [4, 5]. Several interventions and alternative medications have been studied in order to mitigate the undesirable side effects of GCs, such as altering the time course of GC administrations, and the use of selective GR agonists/modulators (SEGRAMs). One study by Salamone *et al*., showed by weekly treatment of GCs rather than daily treatment abrogated GCs adverse effects on skeletal muscle [6]. However, the animals used had no chronic inflammatory condition, and therefore the impact on inflammatory muscle wasting compared to GC-induced muscle wasting was not addressed. Selective glucocorticoids receptor modulators (SEGRAMS) are GR agonists that were designed to reduce undesirable side effects of GR dimerization whilst retaining anti-inflammatory properties [7]. These yielded promising results *in vitro* and *in vivo*, and clinical trials showed promising initial results, with strong anti-inflammatory effects, and reduced side effects in patients with COPD and RA [8, 9]. However, SEGRAMs have not yet progressed passed clinical trials or been approved for clinical

application, and their use over classic GC treatment in clinical settings remains unclear, as their success was limited in other studies [7, 10]. Here, we investigated the potential of another therapeutic target that may be useful in alleviating the common undesirable side effects of GC use, including muscle atrophy, by inhibition of the GC-activating enzyme 11 beta-hydroxysteroid dehydrogenase 1 (11β-HSD1). The enzyme 11β-HSD1 has been shown to play a central role in mediating the actions of both endogenous and therapeutic GCs through the conversion of inactive GCs to their active counterparts [11]. The aim of this thesis was to investigate the role of 11β-HSD1 in GC-driven muscle atrophy in the context of chronic obstructive pulmonary disease (COPD) and rheumatoid arthritis (RA) as these diseases represent CIDs with high prevalence of muscle wasting. We hypothesised that genetic 11β-HSD1 inhibition would reduce or prevent skeletal muscle atrophy through reduced GC signalling in CID-associated muscle wasting. We therefore utilised a mouse model of whole-body genetic deletion of 11β-HSD1 in preclinical models of AE-COPD and RA, to investigate whether systemic application of therapeutic 11β-HSD1 inhibitors would be an appropriate therapeutic intervention in alleviating muscle atrophy in COPD and RA.

7.1 Summary of main findings

Muscle atrophy is a key component of cachexia, which is seen in high prevalence in many disease states, such as RA, COPD and several types of cancer

[12]. We highlight the significant impact of muscle wasting on quality of life and mortality for patients with cachexia in Chapter 2, and the association between muscle wasting and an increase in levels of circulating pro-inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), in both animal models and clinical studies [13-15]. The mechanisms that underpin muscle atrophy in cachexia are detailed, with particular focus on direct effects of inflammation on skeletal muscle through activation of catabolic and anti-anabolic pathways (NF-κB, JAK/STAT, MAPK and SMAD signalling), and indirect effects by which pro-inflammatory cytokines contribute to muscle wasting through dysregulation of other tissue and organ systems, such as HPA axis and GC levels, anorexia, adipocyte and hepatic metabolism [16, 17]. The wavering clinical success of anti-cytokine therapies in cachexia is detailed including putative explanations and highlights the importance of alternative therapies in managing cachexia development. In many chronic inflammatory diseases (CIDs), therapeutic GCs are administered in patients due to their potent anti-inflammatory properties. However, despite their efficacy, their use is associated with unfavourable acute and long-term side effects such as skeletal muscle wasting and osteoporosis [18, 19]. Accordingly, the potential of 11β-HSD1 inhibitors as novel potential therapeutic target in alleviating GCs undesirable side effects is discussed in Chapter 2 [20].

Patients with emphysematous COPD are prone to skeletal muscle wasting, especially during acute exacerbations (AE) of the disease, which correlates with

increased systemic inflammation [21-23]. To assess the potential role of 11β-HSD1 in mediating muscle atrophy during COPD-AE, a mouse model of pulmonary inflammation with emphysema combined with a global genetic deletion of 11β-HSD1 was utilized in Chapter 3. This model is characterised by alveolar destruction and progressive enlargement of airspaces [24], with recruitment of neutrophils, macrophages and lymphocytes to the lungs [25], lung inflammation and systemic inflammatory response with elevated lung and serum IL-6 and IL-1β cytokines [25], and progressive muscle wasting driven by ubiquitin proteosome system (UPS)-mediated proteolysis, increased autophagy and reduced protein synthesis [26, 27]. We selected this model to examine the contribution of 11β-HSD1 to this inflammatory muscle wasting phenotype [26]. Muscle wasting was aggravated in LPS-11β-HSD1KO mice compared to WT animals, with elevated induction of catabolic and anti-anabolic pathways (such as Atrogin-1 and MuRF1 induction) and suppressed anabolic and anti-catabolic signalling (such as p-S6 and p-FoxO) in skeletal muscle. These findings could not be attributed to exacerbated local or systemic inflammation in absence of 11β-HSD1, instead we found serum corticosterone was significantly increased in LPS-11β-HSD1KO mice compared to WT animals. Serum of LPS-11β-HSD1KO mice displayed anti-anabolic activity *in vitro* measured as myoblast-myotube fusion, and this inhibitory effect on post-natal myonuclear accretion was reproduced by corticosterone. Combined 11β-HSD1 inhibition and systemic and local inflammation exacerbated skeletal muscle atrophy and elevated endogenous GC

levels, indicating that the global deletion of 11β-HSD1 lead to a dysregulation of endogenous circulating GCs in this model, resulting in a worsened muscle wasting phenotype. This study revealed a previously unrecognised role of 11β-HSD1 in the regulation of the HPA axis and circulating corticosteroids under conditions of acute lung inflammation and COPD in muscle wasting pertaining to anabolic muscle metabolism.

In Chapter 4, we described the mechanisms that have been postulated to underpin both muscle and bone loss that occur with inflammation and therapeutic GCs. While tissue specific differences exist between muscle and bone that mediate the actions of GCs, considerable overlap in catabolic and anti-anabolic processes driving GC-induced bone and muscle loss are highlighted. Genomic actions of GCs are detailed with focus on glucocorticoid receptor (GR) signalling and trans-activation/trans-repression in response to inflammation, in both bone and muscle. Of note, we reported the interactions of GCs in muscle, where they parallel the actions of inflammatory activation of the NF-κB/FoxO1 pathway to drive catabolic muscle wasting via increased proteolysis and autophagy [28-30]. Importantly this recognised the unknowns in the balance between the GC suppression of muscle NF-κB/FoxO1 signalling their independent induction of this pathway, and what this means for inflammatory muscle wasting in CID patients treated with GCs. A similar paradox was also considered for bone where GCs suppress inflammatory osteoclast (OC) activation whilst also suppressing anabolic activity in osteoblasts (OBs). In

Chapter 5 we used the overexpression of human TNF-α (TNF-tg) model of polyarthritis to examine this directly *in vivo*, characterising the interaction of therapeutic corticosteroids in the context of chronic polyarthritis, and the net actions on anabolic and catabolic bone metabolism. In this TNF-tg mouse model of polyarthritis, disease is characterised by progressive joint destruction driven by synovitis and leukocyte recruitment that result in joint deformity and systemic TNF-α driven inflammatory phenotype [31, 32]. The systemic complications included both inflammatory osteoporosis, and peripheral muscle wasting driven by increased bone resorption by OCs and muscle proteolysis by activation of the UPS [33]. We chose this model to examine the actions of therapeutic GCs in the management of both disease and systemic manifestations of disease in muscle and bone when corticosteroids are administered at therapeutic oral doses as previously described [20]. Following treatment with oral corticosterone (100ug/ml) at an equivalent dose in adult humans of 40mg of hydrocortisone or 10mg of prednisolone [34], WT and TNF-tg animals had significant elevated serum corticosterone levels and TNF-tg mice showed significant reductions in disease activity and reduced circulating IL-6. We found oral GCs reduces inflammatory bone loss in mice with polyarthritis, but drives GC-induced muscle atrophy, with increased catabolic and anti- anabolic signalling. These effects were mimicked in primary muscle cultures treated with TNF-α and corticosterone. These studies revealed a that when given at effective therapeutic doses, GCs could prevent the acute catabolic actions of inflammatory activated OCs

in bone, protecting from inflammatory bone loss but causing a longer loss of anabolic bone formation by OBs, whilst exacerbating inflammatory muscle wasting through induction of proteolytic genes and transcription factors FoxO1, Fbxo32, and Trim63 [33, 35]. This study raised interesting questions as to whether the GC activating enzyme 11β-HSD1 plays a role in this process.

The GC-activating enzyme 11β-HSD1 has been postulated in playing a key role in driving muscle wasting and osteoporosis in the context of the above study, providing strong support to explore the potential therapeutic benefits of modulating this enzyme in this CID models. We used this same experimental TNF-tg model of therapeutic corticosteroid application using animals with global transgenic deletion of 11β-HSD1 to investigate its contribution to the exacerbated inflammatory muscle wasting phenotype that we observed in Chapter 5. Due to the promising effects 11β-HSD1 inhibition seen in protecting against muscle atrophy in GC excess [20], we combined the TNF-tg mouse model used in Chapter 5 with animals with a global deletion of 11β-HSD1 used in Chapter 2. We used the combined model of GC-treated chronic polyarthritis with global 11β-HSD1 inhibition to determine the role of 11β-HSD1 in skeletal muscle wasting in RA in Chapter 6. Despite a reduced anti-inflammatory response in skeletal muscle, deletion of 11β-HSD1 in mice with polyarthritis that were treated with oral GCs showed a partial protection against GC-induced muscle wasting, which was further reflected in a reduced activation of catabolic pathways, indicating 11β-HSD1 deletion prevents muscle atrophy

associated with GC therapy in a model of polyarthritis. This protective effect appeared to be mediated through the direct actions of suppression of circulating therapeutic corticosteroids on the FoxO1 mediated muscle proteolysis and autophagy, and closely mirrored the protection reported by Morgan *et al.,* [20]. Together this thesis reveals a critical interaction between the anti-inflammatory protective actions of endogenous GCs in CID, providing evidence for a key role of 11β-HSD1 in mediating their effects in models of polyarthritis and AE-COPD. However, the nature of this protective role differs substantially between disease setting, mediated by the protective actions of GCs generated by the HPA axis in AE-COPD and local reactivation of endogenous GCs within muscle in arthritis. This was driven by the suppression of inflammatory catabolic muscle wasting pathways.

7.2 Interpretation of main findings

In response to stressors such as inflammation, GCs are released endogenously via activation of the HPA axis in order to elicit their anti-inflammatory effects. Following their secretion and release from the adrenal gland into circulation, GCs bind to the GR present in cells and tissues around the body, potently suppressing inflammation [36]. The immunosuppressive actions of GCs and resulting decline in pro-inflammatory cytokine production feeds back to the HPA axis, resulting in reduced GC synthesis and secretion [37]. Following their isolation in the 1950s, therapeutic GCs are used widely in their treatment of both acute and chronic

inflammation in clinical practice [38]. Therapeutic application of GCs however raises a paradoxical challenge: suppression of inflammation provides symptom relief and may even attenuate disease progression, yet can result in the development of muscle wasting and inflammatory metabolic disease such as Cushing's syndrome [39]. Side effects besides skeletal muscle wasting including visceral obesity, hypertension and osteoporosis are well established in connection with therapeutic GCs long-term use, yet they remain crucial in the treatment of inflammatory disease [40, 41]. Attention and research into the manipulation of GC action has greatly increased in recent years, with some focus on pre-receptor metabolism of GCs at the tissue level by the enzyme 11β-HSD1.

Widely expressed in tissues such as skeletal muscle, bone and macrophages, 11β-HSD1 primarily converts inactive GCs to their active counterparts (cortisone or 11-dehyrocorticosterone into cortisol or cortisone) [42], increasing GC availability in peripheral tissues. Activity of 11β-HSD1 is up-regulated in response to inflammation in a variety of cell types in several models of acute and chronic inflammation [32, 43, 44]. In addition, activation of the GR has also been shown to increases 11β-HSD1 expression and activity independently of inflammation [45]. These increases in 11β-HSD1 activity and expression further increases local active GC availability and GC action [31]. Whilst this increase in local GC availability is important in resolving local inflammation, previous studies have shown prolonged elevated activity of 11β-HSD1 contributes to the GC excess phenotype [46, 47].

Previous studies have shown 11β-HSD1 and GC availability changes in tissues with inflammation. Mice with genetic overexpression of TNF-α develop a progressive polyarthritis phenotype with chronic inflammation and have increased 11β-HSD1 expression and activity in muscle, reflected in increased corticosterone production and mRNA expression [32]. Additionally, 11β-HSD1 has been shown to contribute to tissue GC-amplification in skeletal muscle following treatment with corticosterone, leading to more prominent myopathy [20]. Therefore, we hypothesised the 11β-HSD1 deletion may exacerbate muscle atrophy driven by endogenously produced GCs during chronic inflammation [48], but protect against muscle wasting in models of GC excess by treatment of therapeutic GCs [20].

7.2.1 *The effects of endogenous glucocorticoids during inflammation*

As previously described in Chapter 1 (page 11), acute inflammation primarily involves the innate immune system acting as a first line of defence against pathogens [49], and may be accompanied by high but transient elevations of inflammatory cytokine and chemokine serum levels. Chronic inflammation involves adaptive immune responses and sustained low-grade elevated levels of pro-inflammatory cytokines [50]. Acute and chronic inflammatory responses were modelled in our AE-COPD (Chapter 3) and RA mouse models (Chapters 5&6). Following two intra-tracheal instillations of elastase to induce emphysema, pulmonary inflammation was induced with a single bolus of LPS (PBS control) in both

WT and 11β-HSD1 null mice to induce an acute pulmonary and systemic inflammatory response representing an acute exacerbation of COPD. Inflammatory markers IL-1RA and CXCL2 were elevated in the lungs of mice receiving LPS confirming pulmonary inflammation, in addition to a significant increase in serum IL-6 levels. These elevated levels of inflammatory markers were in line with clinical parameters of an AE-COPD seen in patients [51], and were accompanied by a skeletal muscle wasting phenotype [23]. In our model of chronic polyarthritis similar inductions of inflammatory markers were observed, such as significantly higher serum IL-6 levels in TNF-tg mice (Chapter 5) [33]. Importantly, a notable increase in osteoclast levels in the synovial joint which is indicative of synovial joint destruction, and induction of skeletal muscle wasting demonstrated by reduced wet muscle weights. This illustrates in models of AE-COPD and RA muscle wasting is driven by inflammation both directly and indirectly.

Despite well-established HPA activation in response to systemic inflammation [52], increases in circulating corticosterone levels were not detected in WT emphysematous mice following LPS-induced pulmonary inflammation. In line with previously reported raised circulating levels of pro-inflammatory cytokines in response to IT-instilled LPS [53], elevated serum IL-6 levels were confirmed in the current study. However, HPA-mediated GC release is subject to a strict negative feedback regulation [54], and accordingly, transient elevations in serum corticosterone levels are no longer detectable 48 hours following pulmonary

inflammation in WT mice with emphysema. In support of this notion, muscle expression levels of FoxO, a GR-response gene, were still elevated at this time point and may reflect a lagging response to transient circulating GC levels. Despite no elevated serum corticosterone in mice with polyarthritis (data not published), mRNA expression of GC-responsive gene GILZ was upregulated in skeletal muscle. Clinical studies have shown patients with RA have a marked reduction in systemic cortisol levels compared to healthy controls [55, 56], which was not observed in our model of polyarthritis as GC levels were similar between WT and TNF-tg mice.

7.2.2 The effects of therapeutic glucocorticoids during inflammation

Both COPD and RA are treated with therapeutic GCs in order to supress systemic and local inflammation [57, 58]. Despite their side effects, therapeutic GCs are instrumental in the treatment of CIDs and have continuously shown improvements in disease activity and reducing inflammation in clinical studies [59]. We therefore investigated the effects of corticosterone treatment in the TNF-tg model of polyarthritis with emphasis on skeletal muscle and bone loss. We found TNF-tg mice with GC treatment significantly improved disease activity compared to untreated mice reflected in disease score, in addition to successful suppression of circulating IL-6 levels, reduced osteoclast number in the synovial joint and reduced joint erosion. These data confirm the deleterious effects GCs have on bone metabolism were outweighed by their ability to suppress inflammatory bone loss.

However, their long-term use may still be unsuitable due to the reported suppression in bone formation associated processes. Whilst our study highlighted an important role of therapeutic GCs protecting against inflammatory bone loss, there was a marked increase in muscle wasting in GC-treated polyarthritic mice. These data coincided with previous models of inflammation, with rats receiving synthetic GCs during sepsis showing exacerbated muscle atrophy [60] and reports of a single dose of GCs resulting in muscle wasting in RA patients [61]. Furthermore, treatment with GCs in the TNF-tg mice significantly reduced local skeletal muscle inflammation, reflected in suppressed circulating and peripheral IL-6 levels and muscle CXCL1 expression. This indicates that administration of corticosterone promotes muscle wasting in RA independently of inflammation. In contrast to bone in this model, the deleterious impact of GCs in muscle outweighed their anti-inflammatory effects. Whilst treatment of therapeutic GCs was not investigated in our model of AE-COPD, this will be part of follow-up research to investigate the relative benefit of suppressing inflammation-driven muscle wasting [53], compared to GC-induced muscle loss in this context. Based on prior studies, we might predict that GCs will exacerbate muscle wasting through the induction of muscle catabolism driven by E3 ubiquitin ligases MuRF-1, Atrogin-1 and upregulation of FoxO transcription factors [62, 63], in a similar manner to the TNF-tg model. Clinical studies are complicated in this disease setting where inhaled steroids may have an entirely different mechanism for driving wasting. Previous studies have shown inhaled GCs had little

effect on Type II muscle fibers in patients with asthma over long treatment durations [64], suggesting the mechanisms underpinning therapeutic GC-driven muscle wasting may differ by administration and dose of treatment. The role of 11β-HSD1 in this context remains unclear, but may show a similar degree of protection in muscle as reported *in vivo* [65]. In addition to directing further research into the establishing the lowest effective dose and duration of corticosteroid as a necessity in reducing their undesirable side-effects such as muscle wasting [66], adjuvant treatments with compounds that prevent the catabolic actions of GCs without interfering with their immune-suppressive effects could be considered as novel therapies in CID.

7.2.3 <u>The role of 11β-HSD1 in endogenous GC responses</u>

The enzyme 11β-HSD1 converts inactive GCs to their active counterparts, is up-regulated in response to inflammation and GR activation, and increases GC availability in peripheral tissues which is vital in the resolution of local inflammation [31, 32, 42-45]. In our previous study utilising the 11β-HSD1/KO mouse combined with TNF-tg mice showed worsened polyarthritis in animals null of 11β-HSD1, with marked increases in joint swelling and deformity [67]. This worsened RA phenotype was not attributed to alterations in circulating GCs or inflammation, as serum corticosterone and TNF-α remained constant across TNF-tg and TNF-tg$^{11\beta\text{-HSD1/KO}}$ animals [67]. This study highlighted an important role of 11β-HSD1 in GC activation

of non-mesenchymal cells in RA, of which is attributed to worsened disease activity [67]. In addition, a pronounced muscle wasting phenotype was seen in TNF-tg mice with a genetic deletion of 11β-HSD1, which was attributed to a greater inflammatory muscle atrophy due to the absence of endogenous GC generation by 11β-HSD1 [48]. We therefore used a mouse model of 11β-HSD1 genetic deletion to determine the role of 11β-HSD1 in GC-associated muscle atrophy in AE-COPD and RA. Interestingly, similar effects were seen in our model of AE-COPD, with exacerbated muscle atrophy in 11β-HSD1 null mice receiving LPS compared to WT. However, this worsened muscle wasting was not accompanied by increased muscle inflammation in the muscle, as inflammatory markers CXCL1, IκBα and IL-1β were reduced in 11β-HSD1/KO mice compared to WT with LPS. These differences between WT and 11β-HSD1/KO did not extend to lung inflammation (IL-1Rα and CXCL2), nor serum IL-6. Conversely, serum corticosterone and muscle GILZ mRNA levels were significantly upregulated following IT-LPS in 11β-HSD1/KO animals compared to WT, providing a potential explanation for the reduced muscle inflammatory signalling in 11β-HSD1/KO. Taken together, these data suggest that systemic 11β-HSD1 inhibition worsens muscle phenotypes in both RA and AE-COPD. Whilst we found that compared to WT animals, 11β-HSD1/KO mice showed little differences in body weights in AE-COPD, but in RA 11β-HSD1 deletion worsened disease activity, it is important to recognise the differences in duration of inflammation in both models, with TNF-tg animals having significantly longer exposure to pro-inflammatory

cytokines than AE-COPD mice (48 hours). This could provide an explanation for the worsened disease phenotype seen in our RA model. This may also account for the observed elevation in serum corticosterone seen in AE-COPD that was not apparent in our RA model. Deletion of GR in the pituitary leads to increased GC levels systemically, in addition to a reduced suppression of corticosterone production with GC treatment [68]. These data further support that 11β-HSD1 plays a pivotal role in regulating the HPA axis during times of increased inflammation, both acute and chronic, in the context of endogenous GCs. The deleterious effects seen in skeletal muscle across both models will be discussed further below.

7.2.4 *The role of 11β-HSD1 in therapeutic GC responses*

Therapeutic GCs and their application in treating inflammation are widely accepted in clinical practice, however their long-term use is associated with GC excess and the development of Cushing's syndrome [40], leading to muscle atrophy and osteoporosis [69]. The enzyme 11β-HSD1 increases local activation of GCs [70], leading to increased GR activation, which is necessary for reducing local inflammation, however, previous studies have shown prolonged elevated activity of 11β-HSD1 contributes to the GC excess phenotype [46, 47]. We investigated the role of 11β-HSD1 in RA in driving muscle wasting in combination with corticosterone therapy (Chapter 5). We found that in contrast to endogenous GCs, we saw a protective effect of combined therapeutic corticosteroid treatment and 11β-HSD1

deletion in polyarthritis in muscle. This is in line with Morgan *et al.*, who showed administration of GCs in 11β-HSD1 null mice prevented the deleterious side effects usually observed with GC excess, such as myopathy and glucose intolerance [20]. Although the effects of therapeutic GCs were not examined in our AE-COPD-11β-HSD1/KO model, previous work describes genetic deletion of GR in muscle protects against dexamethasone induced atrophy [71], suggesting that supressing levels of active GCs in muscle by inhibiting HSD may also provide against exogenously applied GCs in the context of COPD-AE. The promising results of inhibition of GC activity in skeletal muscle in case of exogenous GC application paves the way for future research into a combined therapeutic approach. Although GR inhibitors, such as mifepristone, have been researched and developed, their therapeutic application is limited due to whole-body side effects such as GC excess, hypokalaemia (elevated potassium in the blood) and adrenal insufficiency [72]. Therefore, the differential expression of 11β-HSD1 in the liver, adipose tissue, bone and skeletal muscle may allow to direct the effects 11β-HSD1 inhibitors to certain tissues, which may be more suitable at combating the deleterious side effects of therapeutic GCs due to a more targeted approach.

7.2.5 *The role of 11β-HSD1 in inflammation and GC-induced muscle wasting processes*

Cytokines such as IL-1 and IL-6 have been shown to downregulate muscle protein synthesis and myoblast differentiation through IGF-1 inhibition [73]. Elevated levels of TNF-α have also been implicated in upregulating the nuclear factor kappa B (NF-κB) pathway, ultimately increasing muscle proteolysis through increases in E3 ligases Atrogin-1 and MuRF-1 transcription [74]. In our RA study, we found treatment with GCs significantly increased catabolic signalling (FoxO1, Trim63, Fbxo32) and reduced anabolic gene expression (IGF-1), whilst reducing inflammatory gene expression (IL-6, CXCL1) in skeletal muscle. Here, it looks like treatment of therapeutic GCs in mice with polyarthritis drives the exacerbated muscle wasting phenotype through GC-mediated effects rather than inflammatory-driven atrophy.

In TNF-tg$^{11\beta KO}$ mice treated with GCs, a partial protection against GC-induced muscle atrophy is seen. Differences in skeletal muscle during these diseases may offer some explanation to the driving mechanisms that drive the atrophic phenotype, such as exacerbations with acute surges in inflammation versus chronic inflammation. Patients with RA may experience muscle wasting as a result of loss of mobility, due to reduced muscle mass and strength, inactivity due to pain, and increased risk of fractures [75]. Age-related muscle loss known as sarcopenia may also play a role in the muscle wasting seen in RA patients, as generally RA affects an older demographic of people [75]. Whilst some factors implicated in COPD-related

muscle wasting may overlap, such as loss of mobility due to exercise intolerance and weakness, there are distinct features of COPD-related myopathy including reduced expiratory flow and airflow limitation leading to hypoxemia, early occurrences of dyspnea, and hypermetabolism [76, 77].

Global deletion of 11β-HSD1 in mice with chronic polyarthritis have previously shown to have worsened muscle atrophy compared to polyarthritic mice with 11β-HSD1 [48]. Worsened muscle atrophy in RA and AE-COPD with deletion of 11β-HSD1 were primarily driven by increased catabolic signalling (FoxO, MuRF1, Atrogin-1. Anti-anabolic mechanisms underpinning the muscle atrophy phenotype in GC-treated TNF-tg mice were similar to those seen in the AE-COPD 11β-HSD1KO mice in Chapter 3, although in contrast we saw a significant increase in catabolic signalling (Redd1, FoxO1, Atrogin-1 and MuRF-1) in the muscle of polyarthritic mice with GC therapy.

In our model of AE-COPD, pulmonary inflammation induced by LPS resulted in reduced anti-anabolic signalling in addition to a lowered capacity for myonuclear accretion in 11β-HSD1KO mice. Downstream targets of mTOR signalling, including S6, 4E-BP1 and ULK1, showed increased phosphorylation in WT animals receiving LPS compared to mice with genetic ablation of 11β-HSD1, suggesting an attenuated anabolic signalling response in the skeletal muscle of these mice. Deletion of 11β-HSD1 had no enhanced effect on inflammatory signalling within muscle in response to pulmonary inflammation, and therefore we deduced the anti-anabolic signalling

heightened in the 11β-HSD1KO animals were not a result of an increased inflammatory response. During an acute exacerbation of COPD, serum and muscle IGF-1 levels are decreased in patients with cachectic COPD [78, 79]. This was in line with our 11β-HSD1/KO animals receiving LPS, but not in WT animals, further showing the importance of 11β-HSD1 in protecting against atrophy during AE-COPD.

In conclusion, our data demonstrate 11β-HSD1 attenuates endogenous GC-mediated muscle wasting in COPD-AE and RA as its absence aggravates muscle atrophy in models of CID. Conversely, 11β-HSD1 inhibition provides partial protection to muscle wasting responses caused by therapeutic GCs.

7.3 Methodological considerations

Several models are deployed in this thesis to address the effects of endogenous and therapeutic GCs in skeletal muscle wasting, in addition to the role of 11β-HSD1. In our study with a model of acute exacerbation in emphysematous mice (Chapter 2), an acute inflammatory response is triggered by the administration of LPS intra-tracheally into the lungs of mice with emphysema. Previously it has been shown 48-hour post instillation of LPS in emphysematous mice coincides with a peak muscle atrophy response [80] and following that timepoint the resolution of inflammation occurs, and muscle repair and regeneration is activated [80]. Despite this, our findings suggest that muscle recovery responses were already initiated in WT mice 48h following LPS instillation, based on significant increases in anabolic signalling (p-

S6 and p-mTOR). Previous animal studies have shown pulmonary inflammation in an acute setting induces a loss of skeletal muscle that is reversible [81, 82]. As the dynamics of the atrophy and recovery responses in this model of transient muscle atrophy are dictated by the underlying processes studied in muscle lysates, these should be considered with care during their interpretation. Accordingly, the differences in muscle mass between WT and 11β-HSD1KO animals treated with LPS, may reflect a reduced anabolic response in 11β-HSD1KO mice, which may be due to dysregulation of the HPA axis and GC secretion. Whilst our model reflects an acute exacerbation recovery response in emphysematous mice, consideration of the frequency of exacerbations must be taken when discussing these data in the context of endogenous GC levels, and further studies with repeated cycles of pulmonary inflammation in 11β-HSD1/KO animals will offer further insight into the long-term effects of 11β-HSD1 inhibition on pulmonary and systemic inflammation and endogenous GC levels. Animal studies have highlighted a reduced capacity for muscle recovery following frequent exacerbations, with initial muscle catabolism remaining similar during each exacerbation [83]. The majority of acute exacerbations of COPD (59%) are viral or bacterial induced [84], with co-infections of both found in 20-30% of hospitalised AE-COPD patients [85]. Therefore, whilst our model of AE-COPD utilises the bacterial component LPS to induce pulmonary and systemic inflammation, similar studies investigating the role of 11β-HSD1 and GCs in AE-COPD

associated muscle wasting using other pathogens such as viruses may improve translatability to AE-COPD patients.

An important factor to note when discussing the circulating GC levels evaluated in all the murine models is the diurnal rhythm of endogenous GC release via the HPA axis, which may account for slight differences in expected serum corticosterone values. Whilst all experiments were designed with this in mind, subtle differences in expected levels of serum corticosterone may be due to this, and may be counteracted in future studies by ensuring collection times of serum are consistent . Male mice were used in the studies performed in this thesis which is another limitation of study design. Fluctuating levels of gonadal steroids in females across the oestrous cycle are a major factor contributing to sex differences in the HPA axis activity in females compared to males [86], therefore in order to reduce variation levels of HPA axis activity, male mice were used. However, with RA predominantly affecting women [87] and prevalence of COPD continuing to rise in women [88], using female mice in future studies may improve translatability to human patients.

7.4 Translational Implications and impact of results

Experimental models of chronic inflammatory disease and associated muscle wasting may offer insights into the mechanisms underpinning this debilitating aspect and prognostic determinant of CID. Acute exacerbations of COPD are associated with accelerated disease progression and reduced survival [89], however patients with

frequent exacerbations are more susceptible to weight and muscle loss. In addition, exacerbation history in COPD is a predictor of future exacerbations [89-91], further emphasizing the importance of understanding the underlying causes and mechanisms of COPD-AE associated muscle wasting. Previous animal models of acute exacerbations in COPD have shown suppressed anti-anabolic and increased catabolic signalling in skeletal muscle [80], which coincide somewhat with our data with increased catabolic signalling, although reduced anabolic signalling during inflammatory resolution and muscle recovery is less clear. That said, frequent episodes of acute exacerbations in the same mouse model has demonstrated a reduced capacity for muscle recovery [83]. In line with our findings, patients in ICU suffering with acute muscle wasting also showed reduced protein synthesis signalling in skeletal muscle [92].

11β-HSD1 inhibition may not be a suitable therapeutic intervention during chronic inflammatory disease in an endogenous GC setting, however in settings where GCs are applied therapeutically, 11β-HSD1 inhibitors may alleviate the undesirable side effects caused by GCs, such as muscle wasting. In our AE-COPD model we found deletion of 11β-HSD1 combined with pulmonary inflammation significantly increased circulating levels of corticosterone, indicative of a lack of HPA axis regulation. Therefore investigation into the long-term side effects of the use of 11β-HSD1 inhibitors would be required, as consequences for HPA axis regulation

must first be evaluated with previous research indicating prolonged inhibition may interfere with its regulation [93].

7.5 Conclusion and future perspectives

Ultimately, our data suggest inhibition of 11β-HSD1 does not protect against endogenous GC-mediated muscle wasting, independent of the mechanisms underpinning this phenotype across both COPD and RA. However, when 11β-HSD1 inhibition is applied in a setting of therapeutic GCs such as RA, protective effects on muscle wasting may be anticipated. However, this appears to be mediated at the cost of the anti-inflammatory therapeutic actions of corticosteroids and raises the question as to whether the therapeutic inhibition of 11β-HSD1 can be safely administered in human CID without exacerbating disease or causing GC resistance [94]. Instead, the application of 11β-HSD1 inhibitors may be better suited to situations where their levels are increased in the absence of inflammation, such as in Cushing's syndrome or iatrogenic Cushing's [95]. Chronic intake of inhaled GCs have also been implicated in driving muscle wasting in patients with chronic respiratory diseases, which are often prescribed for patients experiencing AE-COPD [96]. Consequently, adjuvant therapies of 11β-HSD1 inhibitors with therapeutic GCs may provide protection against muscle wasting in disease settings such as RA and COPD. These findings could be extrapolated further with the treatment of other acute and chronic inflammatory diseases treated with GCs, such as acute respiratory

distress syndrome seen in Covid-19 patients, but must be carefully weighed up to ensure that protection from GC-induced muscle wasting does not come at the expense of exacerbation of inflammatory disease and therapeutic GC resistance [97].

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Abbreviations

11DHC	11-dehydrocortocosterone
11β-HSD1	11beta-hydroxysteroid dehydrogenase type 1
11β-HSD2	11beta-hydroxysteroid dehydrogenase type 2
11βKO	11beta knock-out
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
ACTH	Adrenocorticotropic hormone
ActRIIB	Activin receptor type IIB
ad lib	*Ad libitum*
ADP	Adenosine 5'-diphosphate
AE	Acute exacerbation
AE-COPD	Acute exacerbation of chronic obstructive pulmonary disease
Akt	Protein kinase B
ALP	Autophagic lysosomal pathway
AMP	Adenosine monophosphate
AMPK	AMP-dependent protein kinase
AP	Activator protein
APCA	Anti-citrullinated protein antibodies
Atg	Adipose triglyceride lipase

Atgl	Autophagy-related gene
ATP	Adenosine 5'-triphosphate
AU	Arbitrary units
BALf	Bronchoalveolar lavage fluid
BMI	Body mass index
Bnip	Protein-interacting protein
BSA	Bovine serum albumin
BV	Bone volume
BW	Body weight
C/EBPδ	CCAAT-enhancer-binding protein delta
cAMP	Cyclic adenosine monophosphate
CBG	Corticosteroid-binding globulin
cDNA	Complementary deoxyribonucleic Acid
CHF	Chronic heart failure
CID	Chronic inflammatory disease
CKD	Chronic kidney disease
COPD	Chronic obstructive pulmonary disease
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein

CSA	Cross-sectional area
Ct	Computed tomography
CXCL	Chemokine (C-X-C motif) ligand
CXCR	C-X-C chemokine receptor
DEX	Dexamethasone
DEXA	Dual-energy X-ray absorptiometry
DMARD	Disease modifying anti-rheumatic drugs
DMEM	Dulbecco’s modified eagle’s medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Cortisone
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid
eIF4E	Eukaryotic translation initiation factor 4E
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ESR	Erythrocyte sedimentation rate
FBS	Fetal bovine serum

FEV	Forced expiratory volume
FLS	Fibroblast like synoviocytes
Fn14	Fibroblast growth factor inducible 14
FoxO	Forkhead box protein O
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gastroc	Gastrocnemius
GBS	Glucocorticoid binding sites
GC	Glucocorticoid
GDF	Growth differentiation factor
GILZ	Glucocorticoid-induced leucine zipper
GLP	Glucagon-like peptide
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive elements
Gy	Gray (unit)
HBSS	Hanks' balanced salt solution
HMGB	High-mobility group protein
HPA	Hypothalamic–pituitary–adrenal
HS	Horse serum
hsp	Heat shock protein

HU	Hounsfield units
IBD	Inflammatory bowel disease
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IGF	Insulin-like Growth Factor
IKK	I-kappa-B kinase
IL	Interleukin-1
IT	Intra-trachael
JNK	C-Jun N-terminal kinase
KLF	Krüppel-like factor
KO	Knock-out
LAA	Low attenuation area
LBM	Lean body mass
LC3B	Light chain 3 beta
LIF	Leukemia inhibitory factor
LLC	Lewis lung carcinoma
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MMPs	Matrix metalloproteinases
MPS	Muscle protein synthesis

MRFs	Myogenic regulatory factors
MRI	Magnetic resonance imaging
mRNA	Messenger Ribonucleic acid
Mstn	Myostatin
mTOR	Mechanistic target of rapamycin
MuRF-1	Muscle RING-finger protein-1
Myf	Myogenic factor
Myog	Myogenin
ND	Not detectable
NF-κB	Nuclear factor kappa B
NR3C1	Nuclear receptor subfamily 3 group c member 1
NS	Not significant
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
P1NP	Total procollagen type 1 N-terminal propeptide
p70S6K1	Ribosomal protein S6 kinase β 1
PAMPs	Pathogen-associated molecular patterns
Pax	Paired box
PBMCs	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PI3K	Phosphoinositide 3-kinases
PI3P	Phosphatidylinositol 3-phosphate
PRFs	Pattern recognition factors
Quad	Quadriceps
RA	Rheumatoid arthritis
RAGE	Receptor for Advanced Glycation
REDD1	Regulated in development and DNA damage responses 1
RLU	Relative light unit
RNA	Ribonucleic acid
ROI	Region of interest
ROS	Reactive oxygen species
RU-486	Mifepristone
S100B	S100 calcium-binding protein B
SCs	Satellite cells
SEGRMs	Selective glucocorticoid receptor modulators
Ser	Serine
STAT	Signal transducer and activator of transcription
sTNFR	Soluble tumor necrosis factor receptors

TA	Tibialis anterior
TB.N	Trabecular number
Tb.Th	Trabecular thickness
TF	Transcription factor
TGF-β	Transforming growth factor beta
Thr	Threonine
TNF-a	Tumor necrosis factor-alpha
TNF-tg	Tumor necrosis factor-transgenic
TRAF	TNF receptor-associated factors
TRAP	Tartrate-resistant acid phosphatase
TV	Tissue volume
TWEAK	Tumor Necrosis Factor-like Weak Inducer of Apoptosis
Ube3a	Ubiquitin protein ligase e3a
UCP1	Uncoupling protein 1
µCT	Micro–Computed Tomography
µL	Microlitre
ULK	Autophagy activating kinase
UPS	Ubiquitin-26 S proteosome system
V.C.	Vehicle control
vLDL	Very low-density lipoprotein

WAT	White adipose tissue
WHO	World health organisation
WT	Wild type

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Curriculum Vitae

Justine Michelle Webster

Education

2018 – Present **PhD in Metabolism and Systems Research.**

University of Birmingham / Maastricht University

Supervisors: Dr. Rowan Hardy (IMSR-UoB) & Dr.Ramon Langen (NUTRIM-UM) – '*The role of pre-receptor glucocorticoid metabolism in atrophy signalling in COPD-associated muscle wasting*'.

2016 – 2017 **MRes Biomedical Sciences and Translational Medicine (Merit)**

University of Liverpool

Supervisor: Dr Mark Morgan (UoL) – '*The effects of Integrin αVβ6 on HER2 bioavailability in breast cancer*'.

2013 – 2016 **BSc (Hons) Biomedical Science, Liverpool John Moores University** **(Class 2:1)**

Accredited by the Institute of Biomedical Science and the Health and Care Professions Council (HCPC).

Research Experience

2018 – Present **PhD in Metabolism and Systems Research.**

The focus on my PhD is investigating the effects of glucocorticoid activation by 11β-HSD1 in muscle wasting in a COPD and chronic inflammatory models. Many skills were acquired and further developed, such as experience working with animal models, DNA/RNA extraction and purification, RT-qPCR, electrophoresis and western blot analysis, histology and imaging, thin-layer chromatography,

immunohistochemistry, primary cell culture, ELISA and statistical analysis. I have also developed and improved my oral and written communication skills.

2016 – 2017 **MRes Biomedical Sciences and Translational Medicine**

Completed three, 12-week laboratory projects involving '*There is reduced cross-talk between αvβ6-integrin and Her2 when Her2 is inactive*', '*The effects of Rab7 on Her2 positive breast cancer cells, with the activation of αvβ6 integrin by LAP*' and '*Activation of αvβ6 integrin causes re-localisation of Rab7 in Her2 positive breast cancer cells*'. Many diverse techniques such as immortalised cancer cell culture, western blot and analysis of results (statistical and comprehensive), LDH assays, determination of protein concentration (Biuret method, BCA protein assay, and using Spectrophotometers (Cecil CE2041 and NanoDrop Lite)), transformation of bacterial DNA, immunofluorescence and the use of confocal microscopes (Spinning disk and Zeiss Airyscan) and mass spectrometry were further developed and/or obtained. I also completed the Home Office Prospective Licensee Training Course (Animal Handling - Category B trained).

2016 **BSc (Hons) Biomedical Science Project**

Final year project '*A comparison of four additives commonly used in food products and their genotoxic effects on Escherichia coli using microbiological-based assays*'**,** where I obtained many techniques, such as bacterial cell culture using aseptic techniques, preparation of culture media, gram stain and substance sensitivity.

2013 - 2016 **BSc (Hons) Biomedical Science**

Correct etiquette within the lab with regards to health and safety and SOPs. The ability to use several non-biochemistry laboratory techniques in other divisions such as microbiology, haematology, histology and toxicology.

Licenses & Certifications

Laboratory Animal Science;	Article 9 Wet op de Dierproeven (Wod), Netherlands.
Issued	September 2019.
Radiation Protection Awareness;	Public Health England. Issued May 2018.
Home office Licence;	PIL A & B Certified. Issued Dec 2016.

Publications

Research Articles

(In review) - **Webster, J. M**., Waaijenberg, Kelsy., Worp, W.R.P.H., Kelders, M.C.J.M, Lambrichts, S., Verhaegen, F., Heyden, B.vdH., Smith, C., Lavery, G.G., Schols, A. M.W.J., Hardy, R. S., Langen, R. (2022). 11β-HSD1 determines the extent of muscle atrophy in a model of acute exacerbation of COPD. Submission to *American Journal of Physiology*.

(Manuscript in preparation) – Maldonado, H., Wolanska, K. I., **Webster, J. M**., Rusilowicz, E. V., MacPherson, I. R., Marshall, J. F., Morgan, M. R. (2022). Integrin αVβ6 regulates HER2 bioavailability and breast cancer invasion via a GDI2/Rab5/Rab7 trafficking sub-network. Submission to *Nature Communications*.

Webster, J. M., Fenton, C. G., Martin, C.S., Fareed, S., Wehmeyer, C., Mackie, H., R. Jones., A. P. Seabright., J. W. Lewis., Y. C. Lai., C. S. Goodyear., S. W. Jones., M. S. Cooper., G. G. Lavery., R. Langen., K. Raza and R. S. Hardy. (2019). Therapeutic Glucocorticoids Prevent Bone Loss but Drive Muscle Wasting When Administered in Chronic Polyarthritis. *Arthritis Research & Therapy*. 21(**1**): 182

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Reviews

Webster, J. M., Kempen, L. J., Hardy, R. S., Langen, R. (2020). Inflammation and skeletal muscle wasting during cachexia. *Frontiers in Physiology*. 11, 597675.

Webster, J. M., Fenton, C. G., Langen, R., Hardy, R. S. (2019). Exploring the Interface between Inflammatory and Therapeutic Glucocorticoid Induced Bone and Muscle Loss. *International Journal of Molecular Sciences*. 20(**22**): 5768.

Conferences

November 2021: Poster presentation at the British Endocrine Society Sfe conference in Edinburgh, UK.

December 2019: Poster presentation at the SCWD Cachexia, Sarcopenia & Muscle Wasting conference in Berlin, Germany.

November 2019: Poster presentation at the NUTRIM Symposium 2019, Maastricht, Netherlands.

November 2019: Poster presentation at the British Endocrine Society Sfe conference in Brighton, UK.

March 2019: Oral presentation at American Endocrine Society ENDO conference in New Orleans, USA.

March 2019: Co-author on a scientific poster presented at American Endocrine Society ENDO conference in New Orleans, USA.

December 2018: Poster presentation at the SCWD Cachexia, Sarcopenia & Muscle Wasting conference in Maastricht, Netherlands.

November 2018: Oral presentation at the British Endocrine Society Sfe conference in Glasgow, UK.

2016: Co-author on a scientific poster presented at MELLORLAB Actin meeting in Bristol, UK.

Awards

- Awarded travel grant from British Endocrine Society of £600 to attend BES Sfe 2021.

- Winner of the 'Basic Science' Poster Award at the Conference on Cachexia, Sarcopenia & Muscle Wasting (SCWD), Berlin, December 2019 with a cash award €250.

- Winner of the NUTRIM Fundamental Poster Award at the NUTRIM Symposium November 2019 with a cash award €250.

- Awarded travel grant from British Endocrine Society in 2018 of £600 to attend BES Sfe 2019.

- Awarded travel grant from British Endocrine Society in 2018 of £600 to attend BES Sfe 2018.

- Awarded travel grant from British Endocrine Society in 2019 of £800 to attend ENDO 2019.

Society Memberships

European Respiratory Society

Society for Endocrinology

The American Endocrine Society

Society on Sarcopenia, Cachexia and Muscle Wasting