# Intercellular communication between macrophages, myoblasts and fibroblasts in the context of rheumatoid arthritis-associated skeletal muscle wasting

by

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## Abstract

Approximately 1% of the global population is afflicted with rheumatoid arthritis (RA), of which over a third experience rheumatoid cachexia, a RA-specific form of skeletal muscle wasting. Current patient research is focused on structural and functional outcomes of rheumatoid cachexia, with limited research focusing on the mechanisms causing this disease and their potential modulation. One of the most vital components to address are the complex cellular interactions occurring within the muscle, and how these interactions are affected by the chronic inflammatory autoimmune disease that is RA.

To elucidate these mechanisms further, multiple models were used in this dissertation. Firstly, a rodent collagen-induced arthritis (CIA) model was used to represent RA. The aims of the first two rodent studies were as follows: (1) to determine the extent of ultrastructural change occurring in multiple muscle types as a result of the inflammatory condition; and (2) to assess different cell types, cytokines and growth factors and their relationships to muscle fibre size and number. Secondly, a novel triple cell (myoblast, fibroblast, macrophage) co-culture model was developed from primary human cell isolates, to mimic rheumatoid cachexia with the use of serum derived from RA and healthy participants. This study aimed to elucidate cellular responses to patient serum, as well as determine the efficacy of bone morphogenetic protein-7 (BMP-7) as a treatment strategy.

The CIA model was a physiologically relevant and accurate model with which to investigate rheumatoid cachexia; several muscle types exhibited reductions in mass and cross-sectional area, in line with clinical reports. Furthermore, increases in fibrosis was reported in all muscle groups, independent of fibre type category. Furthermore, this model allowed for better understanding into the mechanisms of rheumatoid cachexia, with the persistent inflammation in the skeletal muscle contributing to heightened activation – but dysregulation – of muscle regenerative responses, resulting in inability to maintain the fibre size.

Using this cellular profile of arthritis, the triple co-culture model was designed to allow for a human simulation of cellular signalling of co-cultured human primary cells in response to exposure to serum collected from non-RA controls and RA patients.

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Firstly, confirming accurate representation of disease signalling in control and patient serum, RA patient plasma indeed indicated dysregulated IL-6/IL-10 concentrations and a relatively pro-inflammatory state when compared to controls. Assessment of myoblast-, fibroblast- and macrophage-related parameters in the triple co-culture model demonstrated both dysregulated muscle and extracellular matrix formation in response to the treatment non-responding serum. These disease-associated outcomes were corrected/limited in the presence of BMP-7, suggesting a potential beneficial role for BMP-7 in management of rheumatoid cachexia.

In conclusion, this dissertation significantly contributes to our understanding of rheumatoid cachexia by consistently illustrating, across *in vitro* and *in vivo*, rodent and human models, the dysregulation occurring in skeletal muscle as a result of the persistent inflammation that may contribute to the structural and functional outcomes reported in rheumatoid cachexia, as well as how this dysregulation may potentially be addressed via modulation by BMP-7.

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## Opsomming

Ongeveer 1% van die wêreldbevolking word geaffekteer deur rumatoïede artritis (RA). Meer as ‘n derde van hierdie groep lei ook aan rumatoïede kakeksie, ‘n RA-spesifieke vorm van skeletspierverlies. Huidige navorsing is gefokus op strukturele en funksionele uitkomste van rumatoïede kakeksie, met beperkte navorsing wat fokus op die meganismes wat hierdie toestand veroorsaak, of moontlike teenvoeters daarvan. Een van die belangrikste aspekte wat aangespreek behoort te word, is die komplekse sellulêre kommunikasie binne-in die spier en hoe hierdie interaksies beïnvloed word deur die kroniese inflammatoriese outo-immuunsiekte wat RA is.

Om hierdie meganismes verder toe te lig, is verskeie modelle in hierdie tesis gebruik. Eerstens is ‘n knaagdier model van kollageen-geïndusserde artritis (CIA) gebruik om RA te verteenwoordig. Die mikpunte van die eerste twee knaagdierstudies was as volg: (1) om die omvang van artritis-geassosieerde ultrastrukturele skade in verskillende spiere te bepaal; en (2) om die verskillende seltipes, sitokiene en groeifaktore, asook hul verhoudings tot spierveselgrootte en –getal te kwantifiseer. Tweedens is ‘n nuwe drievoudige (mioblast, fibroblast en makrofaag) selkultuur uit primêre sel-isolate van menslike bloed ontwikkel, waarmee rumatoïede kakeksie nageboots kon word deur gebruik te maak van serum afkomstig van gesonde en RA skenkers. Hierdie studie het gemik om die sellulêre reaksie op RA serum toe te lig, as ook om die effek van been morfogenetiese proteïen-7 (BMP-7) as moontlike behandeling te evalueer.

Die CIA model was ‘n fisiologies relevante en akkurate model vir ondersoeke na rumatoïede kakeksie; verskeie spiergroepe het dalings in massa en deursnit-area getoon, in lyn met kliniese verslae. Verder is verhoogde omvang van fibrose in alle spiere gemeet, wat onafhanklik van spierveseltipe kategorie was. Hierdie model het tot ‘n verbeterde begrip van die meganismes van rumatoïede kakeksie bygedra, en het getoon dat die voortdurende inflammasie in die skeletspier bygedra het tot ‘n verhoogde aktivering – maar ongekoördineerdheid – van spiervernuwings prosesse, wat tot ‘n onvermoë om spierveselgrootte te onderhou, gelei het.

Deur gebruik te maak van hierdie selvlakprofiel van RA, is die drievoudige kultuur ontwerp om ‘n simulasie van menslike sellulêre seine in gekweekte normale

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menslike selle in reaksie op blootstelling aan serum van normale of RA skenkers, toe te laat. Eerstens is die akkurate verteenwoordiging van siekte seinoordrag in RA bevestig, met RA serum wat inderdaad ‘n wanregulering van IL-6/IL-10 konsentrasies en ‘n relatief pro-inflammatoriese toestand in vergelyking met gesonde serum getoon het. Assessering van mioblast-, fibroblast- en makrofaag-verwante parameters in die drievoudige kultuur het wanregulering van beide spier- en ekstrasellulêre matriksvorming getoon na blootstelling aan serum van RA pasiënte wat nie op behandeling reageer nie. Hierdie siekte-geassosieerde uitkomste is verder ook gekorrigeer/beperk in die teenwoordigheid van BMP-7, wat op ‘n potensieel voordelige rol van BMP-7 in die bestuur van rumatoïede kakeksie dui.

Ten slotte, hierdie tesis maak ‘n beduidende bydrae tot ons begrip van rumatoïede kakeksie deur herhaaldelik, in *in vitro* en *in vivo* modelle, in mense en in knaagdiere, die wanregulering in skeletspier aan te dui, wat volg op die onopgeloste inflammasie in RA en wat bydra tot die strukturele en funksionele uitkomste van skeletspier in RA. Verder dui tesisdata daarop dat hierdie wanregulering potensieel aangespreek kan word deur behandeling met BMP-7.

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## Dedication

I would like to dedicate this dissertation to my father, Deon Ollewagen. I wish you were here to see the outcome of you calling me Dr Tracey years before this was even an idea.

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## List of Abbreviations

| | |
|---|---|
| α-SMA | α-smooth muscle actin |
| AIA | Adjuvant-induced arthritis |
| AP-1 | Activator protein-1 |
| BCA | Bicinchroninic acid |
| BMI | Body mass index |
| BMP-7 | Bone morphogenetic protein-7 |
| CCL | Chemokine (C-C motif) ligand |
| CCL2 | Monocyte chemoattractant protein-1 |
| CCL3 | Macrophage inflammatory protein 1α |
| CHD | Chronic heart disease |
| CIA | Collagen-induced arthritis |
| COPD | Chronic obstructive pulmonary disorder |
| CRP | C-reactive protein |
| CSA | Cross sectional area |
| DCF | Dichlorofluorescein |
| $dH_2O$ | Distilled water |
| DMARDs | Disease-modifying anti-rheumatic drugs |
| ECL | Entactin-collagen IV-laminin |
| ECM | Extracellular matrix |
| EDL | Extensor digitorum longus |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |

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| | |
|---|---|
| EMT | Epithelial to mesenchymal transition |
| EULAR | European League Against Rheumatism |
| FAPs | Fibro-adipogenic progenitor cells |
| $Fe^{3+}$-TPTZ | Ferric tripyridyltriazine |
| FLS | Fibroblast-like synoviocytes |
| FRAP | Ferric ion reducing antioxidant power |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| HGF | Hepatocyte growth factor |
| HREC | Health Research Ethics Committee |
| HRP | Horse-radish peroxidase |
| Id2 | Inhibitor of differentiation 2 |
| IFN-γ | Interferon-γ |
| IGF | Insulin-like growth factor |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| i-NOS | Inducible nitric oxide synthase |
| IRF | Interferon regulatory factor |
| JAK | Janus kinase |
| LPS | Lipopolysaccharide |
| Mafbx | Muscle atrophy f-box |
| MAPK | Mitogen-activated protein kinase |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MDA | Malondialdehyde |
| MIF | Macrophage migration inhibitory factor |

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| | |
|---|---|
| miRNA | Micro-RNA |
| MMP | Matrix metalloproteinase |
| MnSOD | Manganese superoxide dismutase |
| Murf1 | Muscle ring finger-1 |
| MyoD | Myoblast determination protein 1 |
| NC | Non-arthritic controls/normal controls |
| NF-κB | Nuclear factor kappa-light-chain enhancer of activated B cells |
| NLRP3 | NOD-, LRR- and pyrin domain-containing protein 3 |
| NO | Nitric oxide |
| NSAIDs | Non-steroidal anti-inflammatory drugs |
| PAI-1 | Plasminogen activator inhibitor type-1 |
| Pax7 | Paired box protein-7 |
| PCNA | Proliferating cell nuclear antigen |
| PHF | Primary human fibroblast |
| PHM | Primary human myoblast |
| PI3k/Akt | Phosphatidylinositol 3-kinase/protein kinase B |
| PPAR-γ | Peroxisome proliferator-activated receptor gamma |
| QCT | Quantitative computer tomography |
| RA | Rheumatoid arthritis |
| RATN | Rheumatoid arthritis treatment naïve |
| RATNR | Rheumatoid arthritis treatment non-responding |
| RM | Rheumatoid myositis |
| ROS | Reactive oxygen species |
| Rrbp1 | Ribosomal binding protein 1 |

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| | |
|---|---|
| SC | Satellite cell |
| SST | Serum separating tubes |
| T2T | Treat-to-target |
| TA | Tibialis anterior |
| TBA | Thiobarbituric acid |
| TBARS | Thiobabituric acid and reactive substances |
| TBS-T | Tris-buffered saline + tween |
| TGF-β | Transforming growth factor-β |
| TIMP | Tissue inhibitors of metalloproteinase |
| TNF-α | Tumour necrosis factor-α |
| ZIA | Zymosan-induced arthritis |

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# Chapter 1: Introduction

The most common form of inflammatory arthritis is rheumatoid arthritis (RA), which affects articular structures and the synovium of various diarthrodial joints (Firestein, 2003). In addition to joint damage, a number of patients have presented with body composition changes, including a loss of fat free mass, of which muscle is a major component (Rafaela C.E. Santo *et al.*, 2018). This atrophy is termed rheumatoid cachexia. Rheumatoid cachexia leads to reduced functional capacity and muscle weakness, accelerating morbidity and contributing to accelerated mortality (Walsmith and Roubenoff, 2002). There is currently no standard treatment intervention for rheumatoid cachexia, with the most commonly reported interventions including diet and exercise (Summers *et al.*, 2008), a factor limited by the affected joints.

Given the fact that rheumatoid arthritis is a humoral immunity disorder, it is not surprising that many cellular role players are implicated in various symptoms and pathologies associated with RA and RA cachexia. Considering RA as a whole - while even the aetiology of the disease still requires more in-depth elucidation, much research have focused on immune-related role players and their potential significance in development of RA pathology. For example, it was previously hypothesized that it was unlikely that macrophages play a causal role in RA, except as antigen presenting cells (Michaëlssony *et al.*, 1995; Kinne *et al.*, 2000). However, due to the pro-inflammatory nature and role in destruction of especially the classically activated phenotypes, these cells have been shown to significantly contribute to inflammation and joint destruction in both acute and chronic RA (Kinne *et al.*, 2000). Within the intimal lining of the joint, chronically activated fibroblast-like synoviocytes and macrophages maintain inflammatory processes by activating various cells in the local environment, including T-cells and B-cells, leading to the production of immunoglobulins and rheumatoid factor (Firestein, 2003; Udalova, Mantovani and Feldmann, 2016). The macrophages are the main producers of tumor necrosis factor-α (TNF-α), which, along with interleukin-1β (IL-1β) and TNF-α receptors (TNFRs), have been implicated to have primary roles in the pathogenesis of the disease (Williams, Feldmann and Maini, 1992; Choy and Panayi, 2001; Firestein, 2003; Fionula M Brennan and McInnes, 2008). Therapeutic interventions targeting TNF-α and IL-1 and their receptors support the notion that these cytokines are largely involved in RA, independent of T-cell levels (Firestein, 2003).

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RA-associated cellular infiltration is not limited to the synovium and affects numerous organs over the course of this chronic inflammatory auto-immune disease. Most pertinent to the current thesis topic, persistent pro-inflammatory macrophage infiltration is associated with heightened TNF-α and IL-1β secretion in skeletal muscle – these cytokines are thought to act synergistically to alter the balance between protein degradation and synthesis to promote skeletal muscle atrophy in a condition termed rheumatoid cachexia (Walsmith and Roubenoff, 2002; Londhe and Guttridge, 2015). However, the dysregulated interplay between different cellular role players and/or their secretory products which results in this cachexia, has not been fully elucidated.

Alongside the heightened atrophy seen in chronic inflammatory diseases such as RA, excessive fibroblast infiltration and proliferation results in the excessive deposition of extracellular matrix (ECM) components while degradative enzymes are inhibited, resulting in fibrotic tissue formation surrounding the atrophying fibres (Mann *et al.*, 2011), suggesting significant contributions by other cell types. However, the contribution of these cells to muscle functional loss has not been sufficiently assessed in RA. Furthermore, the excessive fibroblast proliferation may potentially be linked back to the lingering presence of M2b macrophages, which secrete both pro-inflammatory cytokines and ECM-inducing components (Schulert *et al.*, 2016; L. Wang *et al.*, 2019). Both the atrophy and the fibrosis could be responsible for impaired muscle function, an outcome regularly observed in RA patients (Helliwell and Jackson, 1994; Matschke, Murphy, Lemmey, P. J. Maddison, *et al.*, 2010; Ceyhan Dogan *et al.*, 2015; Huffman *et al.*, 2017).

Thus, given the multi-player cellular environment and interactive signalling involved, merely depleting macrophage populations is unlikely to be a realistic solution to RA and similar chronic inflammatory conditions. The pro-fibrotic factors, inflammatory cytokines and oxidative stress enzymes originating from macrophages are all also involved in the overall repair. Indeed, macrophage depletion was shown to result in a delay in the presence of these factors, and ultimately heightened fibrosis and muscle impairment in the later stages of regeneration (Xiao, Liu and Chen, 2016). Evidently, there are complex interactions at play between the different cell types in the skeletal muscle, with all cells required to play their vital roles optimally to ensure the maintenance of a healthy muscle. However, this balance is disrupted in RA and

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therefore needs to be further investigated to develop strategies to combat the debilitating condition of rheumatoid cachexia.

This dissertation will investigate rheumatoid cachexia and the dysregulated signalling occurring within the muscle, as well as investigate the interactions between the different cell types in rheumatoid cachexia. Chapter 2 will present a published in-depth literature review investigating these cellular interactions in chronic inflammatory conditions and propose the mechanisms at work in rheumatoid cachexia. Original data will be presented as three chapters. Chapter 3 will present a published, descriptive, comparative study illustrating the ultrastructural changes in different muscles in a rodent collagen-induced arthritis (CIA) model. Chapter 4 will present published data from the rodent CIA model, illustrating dysregulated immune and myogenic processes in RA muscle, with particularly comprehensive description of different cellular role players involved. Finally, chapter 5 will describe a novel triple co-culture model challenged with serum obtained from human RA patients – both treatment naïve and treatment non-responding individuals – to understand the cellular interactions and aberrant signalling mechanisms at play in rheumatoid cachexia. This interplay is also probed further by inclusion of a potential treatment option. These data chapters will be followed by a synthesis and final conclusions in chapter 6. A full reference list is provided as chapter 7.

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# Chapter 2: Literature Review

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## 2.1 Abstract

Although rheumatoid arthritis affects 1% of the global population, the role of rheumatoid cachexia, which occurs in up to a third of patients, is relatively neglected as research focus, despite its significant contribution to decreased quality of life in patients. A better understanding of the cellular and molecular processes involved in rheumatoid cachexia, as well as its potential treatment, is dependent on elucidation of the intricate interactions of the cells involved, such as myoblasts, fibroblasts and macrophages. Persistent RA-associated inflammation results in a relative depletion of the capacity for regeneration and repair in the satellite cell niche. The repair that does proceed is suboptimal due to dysregulated communication from the other cellular role players in this multi-cellular environment. This includes the incomplete switch in macrophage phenotype resulting in a lingering pro-inflammatory state within the tissues, as well as fibroblast-associated dysregulation of the dynamic control of the extracellular matrix. Additional to this endogenous dysregulation, some treatment strategies for RA may exacerbate muscle wasting and no multi-cell investigation has been done in this context. This review summarizes the most recent literature characterising clinical RA cachexia and links these features to the roles of and complex communication between multiple cellular contributors in the muscle niche, highlighting the importance of a targeted approach to therapeutic intervention.

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## 2.2 Introduction

Rheumatoid arthritis (RA) is a multifactorial disease that affects approximately 1% of the global population. Whilst the exact aetiology of RA is still being elucidated, it is known that both genetic and environmental factors initiate the auto-immune response against the synovium by stimulating several cell types and their cytokine secretome (Gaffo, Saag and Curtis, 2006; Majithia and Geraci, 2007).

RA targets not only the joints, but also the surrounding tissues and their resident and infiltrating cellular components resulting in disability and impaired quality of life (Filippin *et al.*, 2013; Lemmey *et al.*, 2016). In this context, a recent review (Andonian and Huffman, 2020) highlighted the underappreciated status of skeletal muscle and emphasized the significant role of muscle health in the preventing disability and cardiometabolic disease. In RA patients there is a reported 7.4-14.0% decrease in muscle mass compared to matched controls, which frequently occurs with a concomitant increase in fat mass (Lemmey *et al.*, 2016). A recent meta-analysis indicated the presence of rheumatoid cachexia in 15-32% of RA cases (Rafaela C E Santo *et al.*, 2018). Cachexia is defined as a "complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass" (Evans *et al.*, 2008). Cachexia can be diagnosed through the assessment of body composition with either bioimpedance analysis or dual energy x-ray absorptiometry (DEXA) (Masuko, 2014). It may be beneficial to consider additional factors for diagnosis including: anorexia, inflammation, metabolic disturbances, physical output, and quality of life (Argilés *et al.*, 2011).The exact molecular mechanisms involved in rheumatoid cachexia are still unclear, but the most popular hypotheses include reduced physical activity associated with joint pain, pro-inflammatory cytokine-induced catabolism, insulin resistance, insufficient protein ingestion and reduced anabolism (Walsmith and Roubenoff, 2002; Filippin *et al.*, 2013). It is also possible that these different mechanisms occur concomitantly or separately during the different phases of disease. These hypotheses are, in part, based on the links between inflammation, muscle loss and dysfunction that have been elucidated in other diseases as diverse as diabetes and cancer (Frier, Noble and Locke, 2008; Li, Malhotra and Kumar, 2008; Perry *et al.*, 2016; Ato *et al.*, 2019). Although rheumatoid cachexia may share some of the mechanisms, the nature of auto-immune diseases increases the complexity of the inflammatory milieu. Another

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factor to consider is that which neuroinflammation associated with RA presents prior to joint destruction, and the potential effect this may have muscle innervation and the loss of mass and strength, although not sufficiently investigated (Kim and Collins, 1981; Rech *et al.*, 2013). The early onset of inflammation opens the window for earlier treatment targeted at specific mechanisms of rheumatoid cachexia. Earlier intervention could feasibly delay the point where dysregulation is so severe that cachexia becomes clinically evident. It is therefore imperative to determine the causative mechanisms involved in rheumatoid cachexia, to provide the basis for the development of early diagnostic predictors, as well as new preventative and therapeutic strategies targeting the regulatory mechanisms.

A limitation of studies investigating the dysregulation of different cellular role players in rheumatoid cachexia, is that they neglect the complex interaction by focussing on one particular cell type in isolation. Although useful in terms of assessing cellular dysfunction, this does not provide a complete picture of rheumatoid cachexia aetiology. Skeletal muscle is a complex tissue niche, containing several co-existing resident cell types, including myoblasts, fibroblasts and a variety of immune cells, which affect each other multi-directionally via various molecular communication mechanisms.

Due to alterations in the relative distribution and type of immune cells and fibroblasts in different myopathies (Chapman, Meza and Lieber, 2016), a comprehensive, integrative assessment of their availability and intercellular signalling in RA is vital to form a holistic picture of dysregulation and/or muscle pathology. As RA is an auto-immune disease, its inflammatory profile is somewhat unique when compared to other chronic inflammatory diseases (Ross, Powrie and Smith, 2020), so that mere suppression of inflammation – either in circulation or in the synovia - may not be sufficient to prevent RA-associated detrimental effects on skeletal muscle health. Additionally, inflammation contributes to muscle repair processes and pure suppression would therefore impair regeneration (Bencze *et al.*, 2012), as discussed in later sections. Thus, a more comprehensive, integrated understanding of the different role players involved in rheumatoid cachexia is required to further develop treatment strategies.

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This review will provide a brief overview of the known dysregulation and clinical manifestation of rheumatoid cachexia, as well as potential aetiological role players. It is followed by a discussion focussed on integrating the roles of the different cell types present in the muscle niche and their paracrine communication in the context of RA-associated inflammation and muscle de- and regeneration.

### 2.3 The clinical features of rheumatoid cachexia within skeletal muscle

The centrally reported feature of rheumatoid cachexia is the presence of increased levels of pro-inflammatory cytokines (systemic and locally), tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Rafaela C E Santo *et al.*, 2018) – which are also primarily targeted in current treatment modalities. In skeletal muscle, inflammation affects normal protein turnover as well as its response to injury. Both processes involve coordinated remodelling of skeletal muscle tissue with the latter involving the activation, proliferation and differentiation of satellite cells – muscle-specific stem cells (Huffman *et al.*, 2017).

Skeletal muscle mass is easily influenced both positively and negatively, either through hypertrophy or atrophy, both of which are facilitated by signalling pathways involved in protein turnover (Díaz *et al.*, 2018). Hypertrophy of the muscle occurs in response to growth stimuli and an increase in protein synthesis, while muscle atrophy occurs with several pathological states including chronic inflammation, disuse and ageing. Cachexia is a well-known consequence of a number of chronic inflammatory diseases including cancer, chronic obstructive pulmonary disease (COPD), chronic heart disease (CHD), cystic fibrosis and RA, amongst others (von Haehling and Anker, 2010). However, the functional effects of the associated muscle atrophy are underestimated, especially since rheumatoid cachexia exhibits both reduced muscle mass and muscle performance, which may lead to disability, poor quality of life and increased mortality.

The specific aetiological role players that determine rheumatoid cachexia outcome are summarised next.

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### 2.4 Clinical disease severity correlates with loss of skeletal muscle fibre size

Cross-sectional area (CSA) is indicative of the potential for muscle force output which is affected not only by muscle size, but also density, fascicle length and the angle of pennation. Several studies in RA patients and animal models of RA report that RA is characterised by a parallel loss of CSA and strength. An early study reported a simple linear regression between grip strength and muscle CSA for both healthy and RA individuals (Helliwell and Jackson, 1994). In line with this, a rodent collagen-induced arthritis (CIA) model indicated a strong correlation between clinical disease scores (arthritis severity in peripheral joints) and locomotion, which was associated with a significant loss in muscle mass across multiple muscle types (*tibialis anterior* (TA), *extensor digitorum longus* (EDL), *soleus* and *gastrocnemius* muscles) (Hartog, Hulsman and Garssen, 2009). Similarly, another rodent study using the same CIA model demonstrated reduced myofibre CSA of *gastrocnemius* and TA muscles alongside severe arthritic changes including extensive bone erosion and cartilage thinning, within 45 days of onset. In this study, a significant inverse correlation was reported between disease clinical scores and myofibre CSA, highlighting the relationship between disease severity and degree of atrophy (Filippin *et al.*, 2013). Based on these studies, it is clear that a reduction in CSA occurs as a result of RA and is one of the key identifying factors of rheumatoid cachexia.

### 2.5 Cachexia profile is time-dependent and not dependent on pain-associated inactivity

Initially, it was suggested that the atrophy associated with RA occurred as a result of inactivity due to painful joints. However, it has since been observed that muscle atrophy and weakness are already present before pain and swelling affect the physical activity of the RA patient (Costamagna *et al.*, 2015). For example, a reduction in several performance outcomes such as knee extension, grip and trunk extension strengths, as well as overall muscle strength index (29%), were reported in RA patients when compared to controls, at a time point early after clinical onset of RA, and preceding bone mineral density loss (Häkkinen *et al.*, 1999). Patients with stable RA and uncompromised appendicular lean mass exhibited significantly higher body fat percentage and significantly smaller *vastus lateralis* muscle CSA than

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matched controls, as well as a reduction in objective physical function (Matschke, Murphy, Lemmey, P. J. Maddison, *et al.*, 2010). This suggests that CSA is already affected from early stage disease and is not rescued even when patients reach a stable state. However, in another report, a reduction in CSA did not affect contractile properties, activation capacity and concentric force, or power elicited by electrical stimulation - a finding that may indicate some loss in voluntary activation due to pain. Alternatively, since pennation angle was reduced with atrophy, this might explain an improved direction of force transduction with maximal stimulation despite reduced CSA (Matschke, Murphy, Lemmey, P. J. Maddison, *et al.*, 2010). Taken together, these data support the conclusion that skeletal muscle CSA is altered early in disease progression and contributes to loss of function in RA patients. This suggests that at least some compensatory muscle regenerative mechanisms may be activated with time, to counter early cachexia-associated loss of strength. These events at cellular levels remain to be fully elucidated as will be discussed later in this review.

### 2.6 Body composition contributes to the severity of cachexia-induced muscle function deficit

Another potential determining factor in the reduction of muscle strength is the accumulation of intramuscular fat (as indicated by decreased muscle density on quantitative computer tomography (QCT) scans). Low muscle density is indicative of increased myocellular lipid content and fatty infiltration in the interstitial space and is a strong negative indicator of muscle quality. In this context, a cross-sectional study on 60 RA patients indicated associations between a low skeletal muscle fat mass (within individual muscles; includes fat inside myocytes and around the muscle fibres) and more effective performance, including greater speed and *quadriceps* muscle strength (Khoja *et al.*, 2018). Similarly, excessive total body fat (obesity) seems to also be a contributing factor in the decline of muscle function. Kramer *et al.* (2012) demonstrated an inverse relationship between muscle density and body fat mass in the context of RA. The same study reported a correlation between muscle density decline and numerous factors related to the inflammatory profile such as increased age, RA duration, IL-6 and TNF-α levels, as well as circulating endogenous glucocorticoids (Kramer *et al.*, 2012). Taken together, these data

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illustrate the important role of inflammatory activation in determining muscle function and highlight that the combination of metabolic and inflammatory dysregulation adds to the complexity of the disease in RA patients.

### 2.7 The role of skeletal muscle fibre type composition

In terms of muscle type-specific sensitivity to rheumatoid cachexia, reports are varied. Generally, changes in catabolic responses between predominantly glycolytic or oxidative skeletal muscle types have been assessed in several non-RA models of chronic inflammation including burn-injury (Fang *et al.*, 1998), chronic heart failure (Li *et al.*, 2007), and sepsis (Minnaard *et al.*, 2005) with data suggesting that fast-twitch glycolytic muscles are more prone to atrophy. In RA specifically, early patient-based studies have reported the most severe atrophy in type II fibres from the *quadriceps femoris* muscle (Wroblewski *et al.*, 1978; Finol *et al.*, 1988). Of interest to the current review topic, some also reported increased collagen fibril deposition around fibres exhibiting mild necrosis (but not in fibres with severe atrophy or necrosis) (Finol *et al.*, 1988). This is in line with our earlier suggestion that cachectic muscle may activate regenerative counter-mechanisms.

Recent animal studies utilizing the CIA model are however more contradictory. On one hand, assessing the *gastrocnemius* as a predominantly fast-twitch and the *soleus* as a predominantly slow-twitch muscle, the loss of muscle mass in rats was more pronounced in the *gastrocnemius* than the *soleus* (López-Menduiña *et al.*, 2010). Similarly, in female *cynomolgus* monkeys, irregularity in muscle fibre size and more prominent atrophy in type II muscle fibres 5 weeks after initial immunization was demonstrated (Horai *et al.*, 2013). In contrast, no difference in muscle weight and force production deficits between *soleus* and EDL muscles were reported in mice, suggesting that changes in *gastrocnemius* and EDL muscles might differ (Yamada *et al.*, 2009). However, a recent study done by our group assessing the *gastrocnemius, soleus* and EDL muscles in rats, demonstrated a severe (≈60%) loss of muscle mass, but with similar severity in terms of reduction in CSA, left shift of fibre size distribution and histological evidence of fibre degradation (Oyenihi *et al.*, 2019), supporting the argument against muscle fibre type as major role player. Interestingly, atrophy was less pronounced in the *vastus lateralis* muscle, which

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suggests that other factors, such as body posture, distance of muscle from affected joints, or even muscle-specific differences in redox profile – which was detailed in this study (Oyenihi *et al.*, 2019) – may contribute relatively more to final outcome than fibre type itself.

The next sections will provide a more in-depth explanation of the complex signalling from multiple cellular role players contributing to rheumatoid cachexia.

## 2.8 Proinflammatory cytokine-induced protein degradation and catabolism in rheumatoid cachexia

Skeletal muscle is a relatively adaptive tissue and is composed of muscle fibres connected within the extracellular matrix (ECM). Skeletal muscle cells are particularly protein rich and the normally encountered mechanical strains require a basal protein turnover to enable maintenance of structural and functional proteins, adjustments to important regulatory functions and a plethora of adaptive responses. It is important that this process of protein turnover remains balanced, as a small increase in degradation or a decrease in synthesis can result in a reduction in overall cell mass which ultimately leads to muscle atrophy (Walsmith and Roubenoff, 2002). As depicted in Figure 2.1, systemic inflammation promotes protein degradation which leads to the loss of body cell mass, and unfortunately targets predominantly lean muscle tissue (Flores *et al.*, 1989; Dinarello and Wolff, 1993; Roubenoff *et al.*, 1994; Bencze *et al.*, 2012; de Oliveira Nunes Teixeira *et al.*, 2013; Horsburgh *et al.*, 2015).

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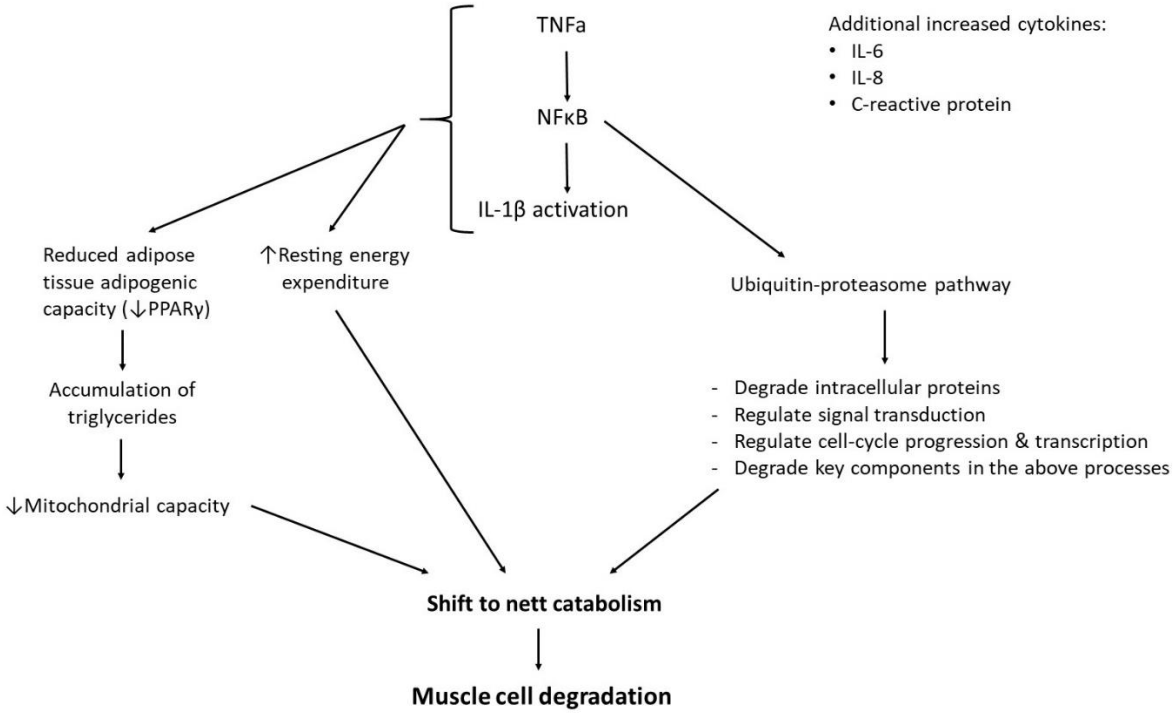


*Figure 2.1: Pathways of proinflammatory cytokine-induced protein degradation in rheumatoid cachexia. TNFα = tumor necrosis factor-α; NFκB = nuclear factor kappa-light chain enhancer of activated B cells; IL-1β = interleukin-1β; PPARγ =peroxisome proliferator activated receptor-gamma.*

Taken together, these studies again indicate that the increase in protein catabolism is largely associated with an increased presence of pro-inflammatory cytokines, which ultimately results in rheumatoid cachexia. The fact that elevated cytokine expression no doubt occurs prior to clinical manifestation of RA joint symptoms, also explains why the loss of muscle mass is already observed in patients early after clinical onset of RA.

## 2.9 Cellular mediators and molecular dysregulation giving rise to rheumatoid cachexia.

Characterisation of muscle pathology in rheumatoid cachexia has gradually progressed since microscopic assessments of different muscle groups in 100 RA patients first revealed muscle fibre atrophy, abundant fibrinoid material and immune cells over 40 years ago (Magyar E, Talerman A, Mohácsy J, Wouters HW, 1977). A

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decade after this first report, the muscle pathology associated with RA was determined to be more complex and was labelled rheumatoid myositis (RM). This was based on electron microscopy evidence of necrosis, varied degrees of atrophy, infiltration of mononuclear cells and myopathic changes. Other reported features included fewer and widely separated myofibrils, the presence of more collagen fibrils (on the myofibre surface and in extracellular spaces) as well as disorientated myofilaments (Finol *et al.*, 1988). These early papers already highlighted the involvement of multiple different cell types in RA pathology. More recent studies further indicate that it is possible for patients with RA to show signs of muscle regeneration. For example, Boutrup and colleagues recently demonstrated that higher myonuclear content in muscle fibres in *vastus lateralis* muscle biopsies taken from RA patients, when compared to healthy controls (Boutrup *et al.*, 2018). This suggests that donation of additional nuclei from the satellite cell pool occurred as a possible compensatory mechanism to preserve muscle health/function.

Below, we review the most relevant literature on relevant cellular role players and their molecular interactions. For clarity of the argument, interactions between skeletal muscle and inflammatory cells are discussed first, in terms of the balance between tissue de- and regeneration. This is followed by a literature overview and discussion of the interplay of fibroblasts and fibrosis in terms of quality of muscle repair.

## 2.10 Satellite cell activation and cell-cell cross-talk in response to inflammation and during regeneration and regrowth

Skeletal muscle has a unique ability to regenerate throughout life due to the presence of satellite cells. Satellite cells reside between the basal lamina and the sarcolemma and in close proximity to capillaries (Nederveen *et al.*, 2016), thus poised to be influenced by the mature muscle cells (fibres), ECM, other cells in the niche and circulating factors. Under healthy, resting conditions, the satellite cells remain quiescent. However, these cells play a major role following injury, at which time they are rapidly activated to proliferate, differentiate and fuse in order to repair the damaged area (Allbrook, 1981). Mechanical loading of muscle with chronic underlying pathology leads to acute damage (Tidball, Welc and Wehling-Henricks, 2018). Hence, skeletal muscle may be required to go through episodic regeneration.

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Over the past decade, another resident interstitial mesenchymal-like stem cell, the fibro-adipogenic progenitor (FAP) cell, has been identified as important in regulating satellite cells (Joe *et al.*, 2010) via engaging in communication with other cells in the satellite cell niche (Lemos *et al.*, 2015). These include immune cells and fibroblasts which play critical roles in all the stages of muscle repair (Yang and Hu, 2018). Figure 2.2 summarises the relationship between the different cell types influencing muscle growth and repair in inflammatory conditions such as rheumatoid arthritis (Allbrook, 1981; Finol *et al.*, 1988; Wüst and Degens, 2007; Arnold *et al.*, 2007; Smith *et al.*, 2008; Joe *et al.*, 2010; Duijnisveld *et al.*, 2011; Heredia *et al.*, 2013; Alway, Myers and Mohamed, 2014; Lemos *et al.*, 2015; Xiao, Liu and Chen, 2016; Little *et al.*, 2017; Kang *et al.*, 2018; Perandini *et al.*, 2018; Yang and Hu, 2018; Biferali *et al.*, 2019; Cappellari, Mantuano and De Luca, 2020; Gudagudi *et al.*, 2020).

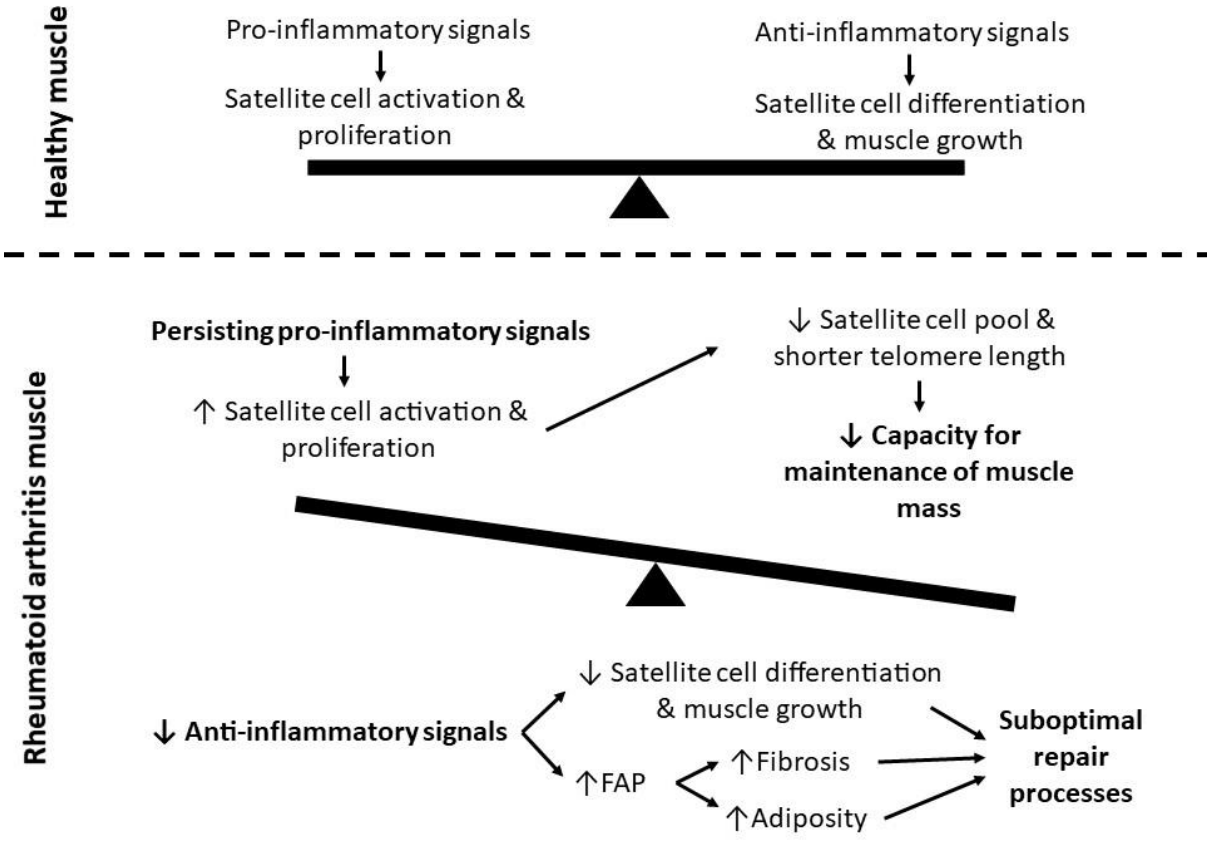

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*Figure 2.2 depicts the imbalance between pro- and anti-inflammatory signalling in RA rodent skeletal muscle, and the resulting influence on muscle repair and growth. (FAP = fibro-adipogenic progenitor cell).*

In a chronic inflammatory setting such as RA, the persistent imbalance of pro- and anti-inflammatory macrophages disturbs normal muscle regeneration by impairing satellite cell proliferation and differentiation (Xiao, Liu and Chen, 2016; Perandini *et al.*, 2018; Yang and Hu, 2018). It is important that the macrophage subtypes work together to maintain the balance between proliferating and differentiating satellite cells. In a rodent model of RA, an imbalance between M1 and M2 macrophages favouring excess M1, has been shown to be related to continued invasion of monocytes into synovial tissue (Misharin *et al.*, 2014). Similarly, macrophage inflitration perpetuates damage in chronic muscle pathologies (Kharraz *et al.*, 2013). It is conceivable that this occurs in the muscle of RA patients, even though the muscle damage is a secondary pathology. Given this evidence of the involvement of macrophages and/or their secreted cytokines as critical contributors to the maintenance of skeletal muscle integrity, a dysregulatory shift in macrophage phenotype can significantly alter the delicate balance between proliferation, differentiation, and fusion of myoblasts to repair or maintain the muscle fibres. In order to better understand the feasibility of therapeutically targeting macrophages in RA, it is necessary to better understand the different immune cell types involved.

## 2.11 A more in-depth review of dysregulated macrophage polarization in RA

In joints with RA, the synovial membrane is also complex in terms of cellular profile, containing activated B- and T-cells, plasma cells, mast cells, and monocytes. Additionally, the presence of activated synovial fibroblasts, chondrocytes, and osteoclasts contributes to cartilage and bone destruction and continued inflammation through the sustained release of cytokines (Fionula M Brennan and McInnes, 2008). These cell types are not limited to the synovial joint and can be found across numerous tissue types, including skeletal muscle (Xiao, Liu and Chen, 2016). Maladaptation, disease-associated pathology or therapeutic interference with the inflammatory response can affect the possibility of synovial joint and muscle repair

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and long-term function significantly. When targeting macrophages for therapeutic effect, the phenotype plasticity of macrophages complicates matters. Temporary ablation of macrophages/monocytes through diphtheria toxin injection after cardiotoxin injury in the TA muscle of mice was reported to attenuate the phenotypic switch of macrophage subsets and ultimately impair muscle regeneration (Wang *et al.*, 2014). Conversely, co-injection of pro-inflammatory macrophages with myoblasts into regenerating mouse skeletal muscle resulted in the generation of twice as many fibres after 4 weeks compared to myoblasts alone or myoblasts with anti-inflammatory macrophages *in vivo*. In this study, subsequent co-culture of myoblasts and macrophages illustrated that co-culture with pro-inflammatory M1 macrophages resulted in an increase in the number of proliferating myoblasts and a decrease in differentiation. The opposite was observed with anti-inflammatory M2 macrophage co-culture (Bencze *et al.*, 2012). This indicates that while persistent inflammation can be detrimental to muscle, and despite the opposing roles of M1 and M2 macrophages, the *synchronised presence* of both phenotypes is vital for optimal skeletal muscle regeneration.

Pro-inflammatory M1 macrophages arise in response to IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and lipopolysaccharide (LPS)/endotoxin in the early stages of muscle repair. Their major role is the removal of necrotic material and to process and present antigens to activate T-cells. M1 macrophages produce pro-inflammatory cytokines TNF-α and IL-1β. The M2 macrophage population is more complex and can be divided into four subtypes, namely M2a, b, c, and d. M2a macrophages are activated by IL-4 and IL-13 and contribute to tissue repair, wound healing and fibrosis in most scenarios of muscle damage. M2a macrophages are most abundant in the later stages of muscle repair (Arnold *et al.*, 2007; Smith *et al.*, 2008) and contribute to fibrosis through the secretion of transforming growth factor (TGF)-β, insulin-like growth factor (IGF) and fibronectin.

In acute damage scenarios, M2a macrophages will switch to M2c macrophages once the inflammatory stimulus is abolished, due to a change in the intracellular cytokine profile. Specifically, M2c macrophages are activated by IL-10 and TGF-β and release anti-inflammatory cytokines, in particular IL-10, to resolve inflammation and limit fibrosis (Tang *et al.*, 2017). However, in scenarios of prolonged or chronic

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inflammation, M2b macrophages are predominantly found. Various signalling factors are involved in the polarisation of macrophages towards a predominance of M2b macrophages, including NF-κB, mitogen-activated protein kinases (MAPKs), PI3K/Akt and interferon regulatory factors (IRFs). M2b macrophages display the capacity to secrete both pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, and anti-inflammatory cytokines such as IL-10 (Edwards *et al.*, 2006; L. Wang *et al.*, 2019), but the factors determining the nett profile of their secretomes are less clear. M2b macrophages are thought to have a role in limiting fibrosis after *acute* cardiac muscle injury (Yue *et al.*, 2020). Recently, micro-RNA (miRNA)-125a-5p overexpression (Schulert *et al.*, 2016) and chemokine (C-C motif) ligand 1 (CCL1) signalling (Sironi *et al.*, 2006) was however implicated in the M2b polarisation seen in juvenile idiopathic arthritis which is associated with poor resolution of inflammation.

Studies suggest that RA patients exhibit persistence of more pro-inflammatory macrophage phenotypes when compared to patients with peripheral spondylarthritis (Vandooren *et al.*, 2009) and osteoarthritis (Tsuneyoshi *et al.*, 2012), where a shift towards an M2 phenotype has been reported. Macrophage depletion using clodronate-containing liposomes prior to initiation of arthritis in a CIA rodent model resulted in reduced M1 cell presence in the joint, alongside a reduction in arthritis disease score, indicating the importance of M1 macrophages in the development of RA (Zhang *et al.*, 2019). However, further investigation is required to fully understand the significance of the M2 subset shifts in RA aetiology. Nonetheless, it would seem that in chronic inflammatory disease, the natural phenotype shift – which occurs via cytokine-induced polarisation – is skewed to limit the complete phenotype transition to achieve anti-inflammatory M2c macrophages. The extracellular signalling factors responsible for this phenomenon, are ideal candidates for therapeutic targeting. (The M2d phenotype is associated with angiogenesis in tumour growth and not relevant to the current topic.)

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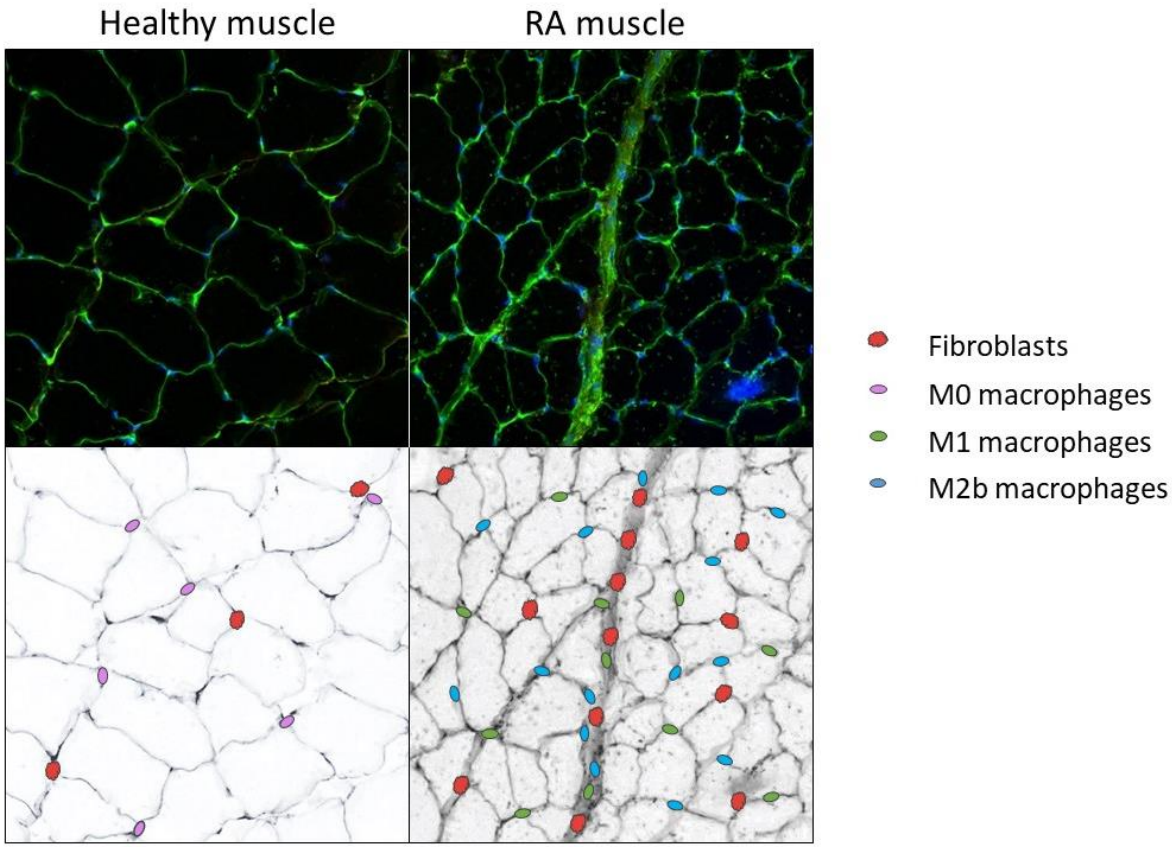


*Figure 2.3: Representative images suggesting the presence of different cell types in healthy versus rheumatoid arthritis skeletal muscle. Fluorescent images from a RA rodent model (study execution described in Oyenihi et al., 2019) indicate clear cachexia and increased fibrosis between muscle fibres. Black and white images indicative of the authors prediction of greater presence of macrophages (M1 and M2b) and fibroblasts in RA.*

## 2.12 Fibroblasts and the extracellular matrix (ECM)

During the regeneration and remodelling phases after muscle injury, structural aspects of muscle are in flux and hence muscle quality may be reduced. Fibroblasts secrete several growth factors and ECM components (fibronectin, collagen I and III, and proteoglycans) during maintenance and repair. Following damage, binding of ECM components (such as fibrin and fibronectin) to the collagens and proteoglycans form a temporary matrix within the injury site. This provides a suitable environment for differentiating myoblasts and a scaffold for regenerating myofibres (Serrano and Muñoz-cánoves, 2010; Mann *et al.*, 2011; Mahdy, 2019).

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Myofibroblasts contribute to tissue repair through wound contracture but may also play a role in the formation of fibrosis. Myofibroblasts were originally thought to be derived only from differentiating resident fibroblasts, however, they can also arise from parenchymal epithelial cells through epithelial to mesenchymal transition (EMT) (Wynn, 2008; Mann *et al.*, 2011) and from FAPs (Serrano *et al.*, 2011). For optimal repair, these cells are present in damaged muscle only transiently, with a rapid reduction in their numbers in the regenerating area. However, in chronic situations, the persistence of FAPs and their differentiation into myofibroblasts and adipocytes results in intramuscular fibrofatty infiltration (Serrano *et al.*, 2011; Lemos *et al.*, 2015). Here, macrophage dysregulation comes into play: the increase in TGF-β secretion by M2 macrophages blocks the TNF-α-induced apoptosis, resulting in prolonged FAP survival. Although not directly assessed in RA, the increased muscle fat mass reported in RA suggest that these processes also contribute to RA myopathy.

Degradation of the temporary ECM occurs after sufficient repair to allow for the optimal growth of the regenerating fibres (Wynn, 2008; Biferali *et al.*, 2019). Matrix metalloproteinases (MMPs) produced by damaged myofibres and infiltrating cells play a key role in the degradation of this temporary ECM (Lemos *et al.*, 2015; Alameddine and Morgan, 2016). However, the excessive deposition of ECM and impaired degradation via TGF-β-dependent mechanisms leads to interstitial fibrosis and subsequent loss of tissue architecture and function. Within the skeletal muscle, excessive ECM deposition, especially collagens, results in impaired muscle fibre regeneration and increased susceptibility to re-injury, ultimately causing morbidity and mortality (Wynn, 2008; Mahdy, 2019).

Macrophages are attracted to the damaged tissue by chemoattractant cytokines and secrete cytokines that indirectly stimulate the production of ECM components, as summarized in Figure 2.4. M1 macrophages release TNF-α and IL-6 which stimulate the proliferation of fibroblasts and FAPs (Bersini *et al.*, 2018), but upon binding to excess fibrinogen also increase the production of IL-1β and TGF-β (Vidal *et al.*, 2008). TNF-α and IL-1β increase the synthesis of collagenases contributing to the destruction of the cartilage in RA (Dinarello and Wolff, 1993; Smith *et al.*, 2008; Perandini *et al.*, 2018). M2a macrophages play a profound role in fibrosis due to the fact that they release a large range of pro-inflammatory molecules such as TGF-β,

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fibronectin, several TIMPs and CCL17 (Mann *et al.*, 2011; Perandini *et al.*, 2018). In addition, both CCL3 (macrophage inflammatory protein 1α) and CCL2 (monocyte chemoattractant protein-1) have been highlighted as promotors of fibrosis through their chemotactic properties (Wynn, 2008). To add to this, fibroblasts were shown to have the capacity to promote arthritis through the production of GM-CSF, which enhances the survival of neutrophils and macrophages (Udalova, Mantovani and Feldmann, 2016), thus further strengthening the inflammatory response in a vicious cycle, much like the self-propagating oxidative damage-inflammation cycle that also comes into play in chronic diseases such as RA (Oyenihi *et al.*, 2019). Disturbance in the balance between classically activated (M1) and alternative (M2) macrophages therefore leads to excessive TGF-β production, resulting in excessive activation of fibroblasts and inhibited apoptosis of FAPs, resulting in excessive ECM production and fibrosis (Perandini *et al.*, 2018; Mahdy, 2019). Related to this, it was suggested by Khoja (Khoja *et al.*, 2018) that the accumulation of fat around the muscle spindles may lead to a similar effect with thickening of the capsule and fibrotic changes in the intrafusal muscle fibres. This has not yet been sufficiently assessed in the context of RA, warranting further research in this context.

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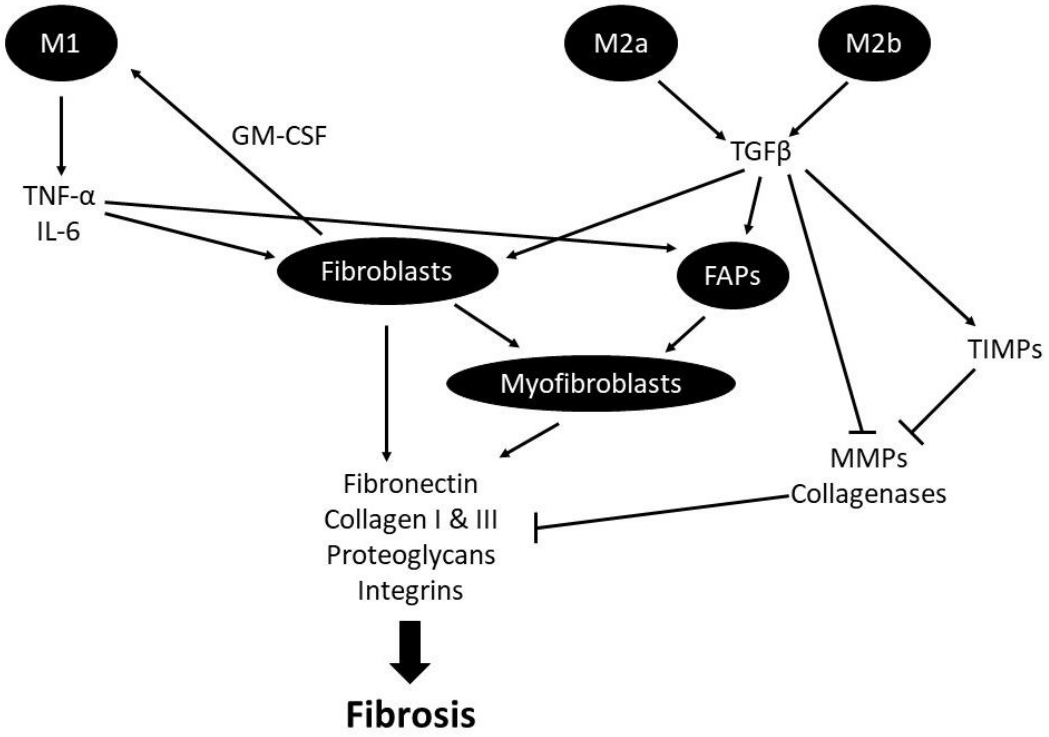


*Figure 2.4: Summary of the interaction between macrophages, fibroblasts and FAPs in the development of tissue fibrosis. TNF-α = tumor necrosis factor-alpha; IL-6 = interleukin-6, TGF-β = transforming growth factor-beta; FAPs = fibro-adipogenic progenitor cells; TIMPs = tissue inhibitor of metalloproteinase; MMPs = metalloproteinases.*

One of the most important factors involved in tissue healing and fibrosis is TGF-β. This profibrotic growth factor is present in skeletal muscle following injury and in dystrophic muscle, where it stimulates fibroblasts to produce ECM components (Zhou *et al.*, 2006; Serrano and Muñoz-cánoves, 2010). During regeneration, the degradation of the initial scaffold of ECM contributes to the generation of protein fragments that mediate biological activities involved in normal tissue repair (Chen and Li, 2009; Mann *et al.*, 2011; Serrano *et al.*, 2011). However, TGF-β reduces the production of enzymes (such as collagenase) and stimulates the production of tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitor type-1 (PAI-1), thereby inhibiting the degradation of the ECM (Zhou *et al.*, 2006; Serrano *et al.*, 2011) and disturbing the final outcome.

Gene expression analysis of skeletal muscle samples from RA patients demonstrated a correlation between disease activity and disability and increased

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concentrations of amino acid precursors to muscle fibrosis (Huffman *et al.*, 2017). Analysis of blood samples from patients with active RA highlighted differences in cell-cell interactions, altered EMT, and increased TGF-β proteins suggestive of increased *de novo* ECM synthesis and fibrosis (Brink *et al.*, 2019). Fibrosis has been demonstrated in several tissues from RA patients and rodent studies. For example, increased fibrous tissue deposition was demonstrated in the joint of RA rodents 6 days post induction with fluorescent visualization of collagen III and fibrinogen (Beziere *et al.*, 2019). Similarly, research from our group has demonstrated a significant increase in collagen accumulation within the *vastus lateralis*, *soleus* and *gastrocnemius* muscles in CIA rodents compared to that of control rodents (Oyenihi *et al.*, 2019), as well as impaired molecular remodelling with fibrotic deposition and impaired cardiomyofibre contractile function (Pironti *et al.*, 2018). In humans, the presence of subclinical myocardial fibrosis resulting from low grade chronic inflammation in a large number of RA patients, has been linked to heart failure in approximately 3.9% of patients (Lazúrová and Tomás, 2017). Furthermore, pulmonary fibrosis is often associated with RA as a result of increased TGF-β and Smad signalling leading to an increase in collagen deposition within the lung tissue (S. Wang *et al.*, 2019). The assessment of fibrosis in RA skeletal muscle tissue is limited, however in chronic inflammation, macrophages expressing both pro- and anti-inflammatory cytokines (TNFα and TGF-β) were reported to have a reduced ability to clear FAPs from the damaged tissue (Lemos *et al.*, 2015). Cross-talk between these cells and satellite cells also occurs via TGF-β. *In vitro*, it has been demonstrated that the isoform of TGF-β has an influence on the satellite cell response (Schabort *et al.*, 2009). Therefore, a full understanding of the role of TGF-β in RA may require that level of sophistication, which can be achieved *in vitro* in single cell culture or co-culture. In chronic kidney disease, chronic inflammation resulted in increased muscle collagen content which also correlated with increased abundance of FAPs (Abramowitz *et al.*, 2018).

To further complicate matters, *in vitro* studies have demonstrated that satellite cells themselves also secrete factors that regulate ECM gene expression independent of TGF-β (Fry *et al.*, 2014, 2017). The use of Pax7 knock-out mice indicated that satellite cells are critical to limit ECM deposition and prevent fibrosis in the first week of regeneration, potentially through a mechanism involving microRNA and exosomes

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circulating the skeletal muscle. For example, microRNA-206 (miR-206) is highly expressed in satellite cells and satellite cell-derived exosomes; it performs its actions through binding to and inhibiting ribosomal binding protein 1 (Rrbp1), a master regulator of collagen synthesis. Knockdown of miR-206 resulted in the increased expression of collagen genes in fibrogenic cells (Fry *et al.*, 2017), proposing a potential mechanism of fibrogenic pathogenesis in chronic inflammatory disorders such as RA. The potential for therapeutic or preventative intervention at this level warrants further investigation.

### 2.13 Finding therapeutic targets in the context of RA

From the literature presented above, it is clear that complex, aberrant intracellular communication resulting from auto-immune activation – and thus persistent inflammation - results in the complex syndrome of rheumatoid cachexia.

We present a predictive image of the muscle niche in and a visual summary of the literature in Figure 2.5. In our opinion, the main site of dysregulation in RA, is the incomplete phenotype switch from M1 to M2c macrophages, which is reflected by an “indecisive” M2b phenotype which can signal either pro- or anti-inflammatory, depending on signalling from its extracellular environment, but does either ineffectively. This has several knock-on effects, such as the dysregulation of the ECM, which limits satellite cell activation and function, as well as contributing to fibrosis. Both these outcomes then further affect other cell types in a self-reinforcing chronic cascade resulting in the increased fat and collagen deposition, and loss of muscle fibre maintenance observed in rheumatoid cachexia.

In our opinion, there are two main avenues to follow for therapeutic intervention. Firstly, an obvious therapeutic strategy would be to prevent the incomplete M1-M2 transition of macrophages. Secondly, modulation of the RA ECM would not only correct satellite cell functionality, but also improve mature muscle fibre and whole muscle functionality and force output, both of which would significantly contribute to complete repair and return to function. A specific target for this approach would be to reduce the proliferation of fibroblasts, present in the affected muscle as well as the affected joint. However, fibroblasts do not act alone in the development of fibrosis,

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which is also stimulated by FAPs and macrophages. Of course, given the complexity of the disease and interplay of contributing cell types – especially once properly established – it would be naïve to consider intervention at any one site in isolation.

The fact that rheumatoid cachexia occurs secondary to primary auto-immune disease, and that inflammation itself is a systemic phenomenon rather than a local one, adds complexity to the quest for a therapeutic strategy. However, recent advances in drug delivery systems and nanotechnology may hold promise for delivery of modulating factors into the muscle ECM. Due to the greater presence of macrophages in affected tissues, the proposed use of drugs encapsulated by phagosome-arrested macrophages to deliver treatment to the desired site is a promising one (Visser, Van Staden and Smith, 2019). Additionally, the use of macrophages to deliver satellite cells to the affected muscle holds promise in promoting skeletal muscle regeneration (Visser and Smith, 2018).

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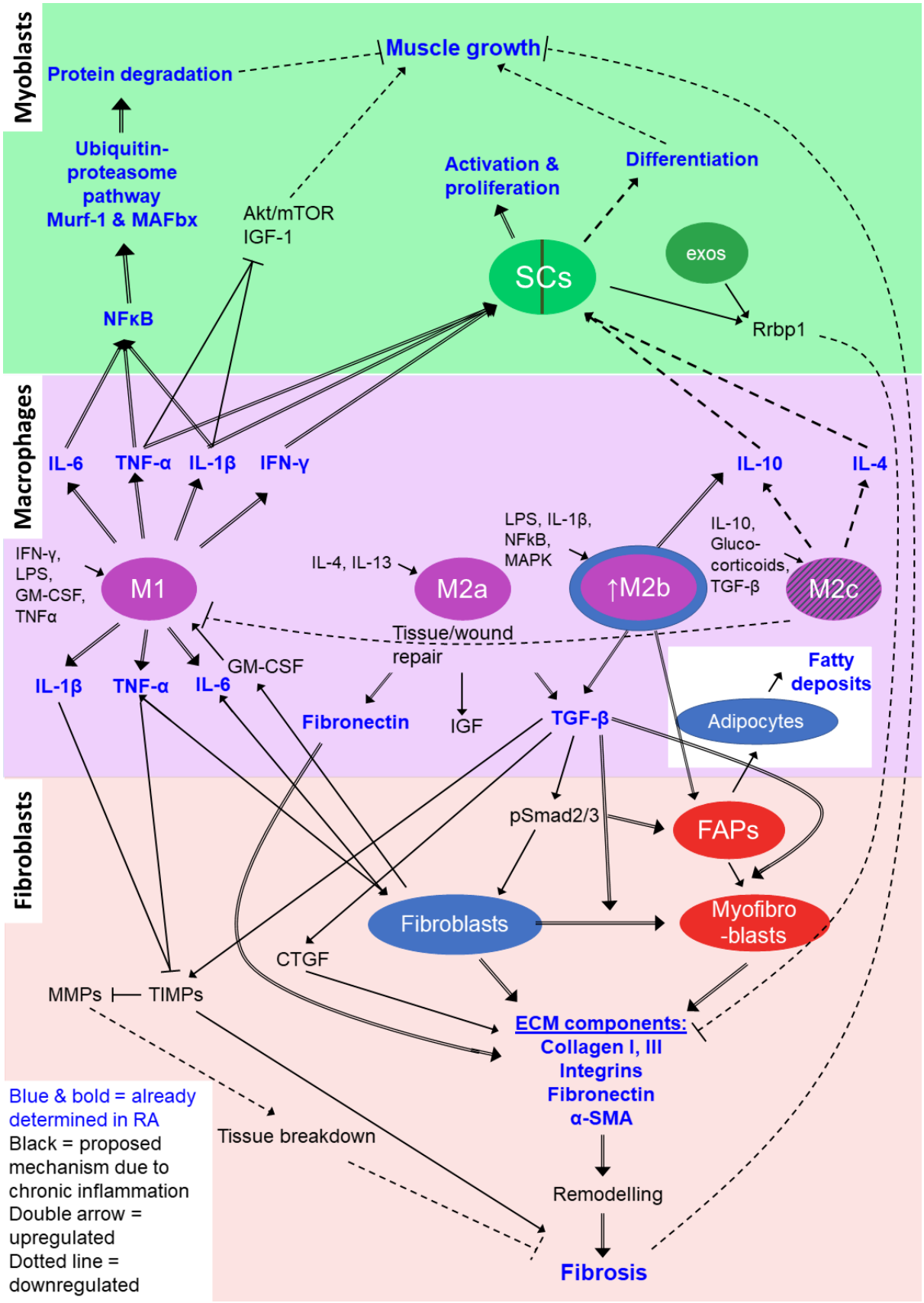


*Figure 2.5: Summary of the cellular interactions in chronic inflammation leading to a decline in muscle growth. The incomplete switch from M1 to M2c results in a greater*

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*presence of M2b macrophages which present both impaired pro- and impaired anti-inflammatory properties, often resulting in enhanced deposition of ECM components, and impaired satellite cell function. This also results in a reduced presence of M2c macrophages. The mechanisms and pathways presented are based on chronic inflammatory microenvironments (proposed in RA), with blue indicating mechanisms confirmed in studies of RA. Different colours indicate different cell focus areas; green = muscle niche; purple = inflammatory system; red = fibroblasts and fibrosis. Increased signalling are indicated by double-line arrows, while dotted line arrows indicate decreased signalling.*

*SCs = satellite cells; IGF = insulin-like growth factor; Murf-1 = muscle ring finger protein-1; Mafbx = muscle atrophy f-box; exos = exosomes; Rrbp1 = ribosome binding protein-1; IL = interleukin; TNF-α = tumor necrosis factor-α; IFN-γ = interferon-γ; LPS = lipopolysaccharides; GM-CSF = granulocyte-monocyte colony-stimulating factor; TGF-β = transforming growth factor-β; TIMP = tissue inhibitor of metalloproteinase; MMP = matrix metalloproteinase; CTGF = connective tissue growth factor; FAPs = fibro-adipogenic progenitor cells; ECM = extracellular matrix; α-SMA = α-smooth muscle actin.*

### 2.14 Are current treatment strategies failing?

Perhaps due to the complexity of RA as a condition (and even the lack of clarity regarding its precise aetiological trigger), as well as the limitations to be overcome in the development of targeted therapeutic approaches, therapeutic strategies for RA still have ample room for improvement. The main therapeutic goal of current treatment strategies is to induce sustained clinical remission or to maintain a low-inflammatory activity of the disease if remission is not possible (Narváez, 2016). This section will give an overview of the current treatment strategies and briefly outline which of these strategies shows potential in terms of targeting the sites we have identified, in the context of delaying or limiting the extent of rheumatoid cachexia.

Popular treatment options for RA vary from the use of non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, disease-modifying anti-rheumatic drugs (DMARDs), and more recently, biological-response mediators (Narváez, 2016;

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Drosos, 2020). The treat-to-target (T2T) approach has been found to be more effective than specific treatment strategies and incorporates aspects such as tight control and monitoring of disease and its biological side effects, and therapeutic adjustments when set targets are not met (Drosos, 2020). NSAIDs and glucocorticoids are considered a first-line therapy and act to rapidly reduce pain and swelling in the affected joints by transiently reducing the inflammation present. The use of both NSAIDs and glucocorticoids are only recommended as short-term treatment or as bridge therapy while the DMARDs gain sufficient effectivity, as they neither slow the progression of RA, nor have disease modifying effects and are linked to a large number of adverse effects (Gaffo, Saag and Curtis, 2006; Kesharwani *et al.*, 2019; Smolen *et al.*, 2020). Currently, the drugs of choice for the treatment of RA are DMARDs (Kesharwani *et al.*, 2019). DMARDs are slower acting compounds that improve symptoms and slow progression of RA. DMARDs are split into two, namely synthetic DMARDS and biological DMARDS (Drosos, 2020; Smolen *et al.*, 2020). The most commonly used DMARD is methotrexate, which has several mechanisms of action. It has the ability to inhibit the proliferation of cells, including inflammatory-cell mediators and lymphocytes, as well as to reduce TNF-α and IL-1β expression, hence its anti-inflammatory effect (Quéméneur *et al.*, 2003; Maksimovic *et al.*, 2020). Studies on methotrexate have also indicated a reduction in the accumulation of toxic compounds such as polyamines that contribute to tissue damage (Cronstein, 2005) as well as a reduction in reactive oxygen groups in the synoviocytes obtained from RA patients (Sung *et al.*, 2000). The European League Against Rheumatism (EULAR) still recommends that immediate treatment with DMARDs should occur upon RA diagnosis, and despite the fact that newer therapeutics have been developed, methotrexate should be the first treatment option (Smolen *et al.*, 2020). Whether methotrexate would be beneficial in rheumatoid cachexia is unknown. Investigation into the effects of methotrexate on satellite cells and skeletal muscle is lacking in the RA model and treatment was unable to shift macrophage phenotype to M2 macrophages once polarised to the M1 phenotype in an *in vitro* model (Shiratori *et al.*, 2018). The effect of methotrexate on fibrosis is also less desirable - the drug were demonstrated to be pro-fibrotic in both liver cells *in vitro* (Prestigiacomo *et al.*, 2017) and in liver tissue of RA patients (Lertnawapan, Chonprasertsuk and Siramolpiwat, 2018). While this treatment may be beneficial and

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used as a first option therapy for RA, it is not necessarily beneficial in treating or preventing the development of secondary symptoms such as rheumatoid cachexia.

In terms of biological-response modifiers, scientists have largely focused on suppression of systemic inflammation, developing therapies targeting specific soluble or cell-surface molecules with the use of monoclonal antibodies and receptor constructs. One of the most successful targets is TNF-α inhibition (adalimumab, etanercept, and infliximab) - these medicines are often used in conjunction with methotrexate (Smolen *et al.*, 2007; Curtis and Singh, 2011; Kesharwani *et al.*, 2019). Etanercept targets TNF-α type II receptor-IgG1 fusion protein while infliximab and adalimumab are specific TNF-α monoclonal antibodies. The use of infliximab with methotrexate significantly improved symptoms of RA including joint swelling, pain, joint damage progression, and CRP concentration compared to that of methotrexate alone in a study assessing 428 patients with active RA (Lipsky *et al.*, 2000). However, in this study there was still a high rate of adverse events, with the most common being infections – which likely resulted from the blanket approach to immune suppression. The use of anti-TNF-α (adalimumab) treatment reduced the diseased joint structural progression compared to methotrexate, which was further reduced by use of the both drugs in combination in a study on 799 patients with active early RA (<3 years since diagnosis) (Breedveld *et al.*, 2006). However, there are still patients who do not respond to the anti-TNF-α therapy. Additional biologics treatment approaches include the blocking of IL-6 through anti-IL-6-receptor monoclonal antibodies (Emery *et al.*, 2008), anti-B-cell therapy (Edwards *et al.*, 2004), and down-regulation of T-cell activation through the modulation of the co-stimulatory signal necessary for activation (Majithia and Geraci, 2007; Blair and Deeks, 2017). Due to many cytokine receptors signalling via Janus kinases (JAKs), another more recent treatment strategy involves JAK inhibitors. JAK inhibitors can be used to determine the effect of inhibition of several cytokines; these include IL-6, GM-CSF, type 1 interferons, IL-7, IL-15, and IL-21 (McInnes and Schett, 2017). While still in the earlier stages of investigation, tofacitinib/methotrexate combination treatment has demonstrated suppressive effects on T-cells, B-cells and fibroblast-like synoviocytes, as well as reductions in MMPs in 15 RA patients compared methotrexate-treated controls (Boyle *et al.*, 2015).

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Despite the advancement of therapeutic strategies to reduce RA disease progression and/or manage pain, not all of these are beneficial in the context of muscle health. While the cachexic effects of the drugs have not been sufficiently investigated in RA patients specifically, their commonly known pathways can be extrapolated to the RA context. For example, glucocorticoids decrease muscle anabolism and increase muscle catabolism through different pathways, including the myostatin pathway, the IGF-1-PI3K-Akt pathway, and the NF-κB pathway (Hanaoka, Peterson and Crofford, 2012) – thus, in particular patients showing significant RA cachexia, this treatment should be avoided or at least paired with therapy that may counter these undesired outcomes. In contrast, the use of DMARDs may also limit sarcopenia due to its inhibition of cytokines such as TNF-α and IL-6. However, DMARDS have been linked to increased body weight (Tournadre *et al.*, 2017) - given the negative effects of muscle fat deposition commonly seen in rheumatoid cachexia, this problem may be exacerbated by these treatments.

Research on specific therapeutic strategies targeting rheumatoid cachexia are limited. Current therapeutic strategies to target cachexia include increasing physical activity, especially in the form of resistance exercise, and dietary alterations, such as a Mediterranean diet supplemented with omega 3 and vitamin D (Tedeschi and Costenbader, 2016) and antioxidant supplements (Bala, 2017). Low IGF-1 expression is associated with lower appendicular lean muscle index and muscle CSA in RA patients (Baker *et al.*, 2015). The use of IGF-1 treatment increased body weight and *gastrocnemius* weight, as well as inhibiting the CIA-induced increase in atrogin-1 and MuRF1 expression in rodents (López-Menduiña *et al.*, 2010). However, IGF-1 is associated with increased cancer risk (Cao *et al.*, 2015). Treatment with a peroxisome proliferator-activated receptor-α (PPARα) agonist had similar effects, attenuating the decrease in *gastrocnemius* weight and fast-twitch myofibre size, and preventing the arthritis-induced increase in atrogin-1 and MuRF1 expression in a rodent CIA model (Castillero *et al.*, 2011). In addition to positive effects in skeletal muscle, PPAR-α also has anti-inflammatory effects and studies have determined that treatment with PPAR-α agonist resulted in reduced oedema and arthritis score in arthritic rodents (Koufany, Jouzeau and Moulin, 2014). However, the use of PPAR agonists are not an option due to their many side effects,

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including congestive heart failure, bone fractures, liver disease and myopathy (Bortolini *et al.*, 2013).

A number of studies have explored the idea of using physical activity and exercise training to improve muscle strength and mobility in RA patients. Regular range of motion exercise over a 2 year period significantly improved strength, disease activity, and physical function, which was further improved in RA patients undergoing 2 years of dynamic strength training (Hakkinen *et al.*, 2001). A 5-week rehabilitation exercise program improved quadriceps strength and activation, and reduced subjective disability without exacerbating disease activity, improvements which were maintained at a 6 month follow up session (Bearne, Scott and Hurley, 2002). Twelve weeks of moderate intensity pool exercise, comprising of a variety of strength, endurance and flexibility exercises, improved grip force and muscle function despite not improving aerobic capacity in RA patients (Bilberg, Ahlmén and Mannerkorpi, 2005). During a 6-week hand exercise programme, RA patients responded similarly to healthy controls, with an increase in hand force measurements, hand function, and increased CSA in the *extensor digitorum communis* muscle (Brorsson *et al.*, 2009). While the majority of these studies have demonstrated beneficial effects on muscle strength and function, few of them report on the cachexia outcomes.

## 2.15 Conclusion

This review highlights the complexity and multi-directionality of cellular interplay in RA cachexia progression. More investigation is required to determine the specific interaction between the cell types mentioned, as limited research has focused on these pathways in terms of rheumatoid cachexia. Review of current treatment strategies illustrates that blanket-approach systemic anti-inflammatory intervention is effective to a degree in RA, but not without side effects or missing the impact on cachexia. The severe impact of rheumatoid cachexia on long term patient independence highlights the importance of addressing not only the primary auto-immune disease, but also its secondary debilitating conditions. Further research is required to develop a more specifically targeted treatment approach, potentially making use of more specific, controlled delivery systems that may be incorporated into T2T approaches to limit rheumatoid cachexia development.

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# Chapter 3

This chapter has been published in Oxidative Medicine and Cellular Longevity (impact factor: 6.54). Although it was a joint study by ABO and TO, the roles of these contributing authors were distinctly different. ABO characterised the CIA model in terms of redox changes. TO was responsible for all aspects of muscle related investigations.

Citation reference: Oyenihi, AB., Ollewagen, T., Myburgh, KH., Powrie, YSL., Smith, C. (2019). Redox status and muscle pathology in rheumatoid arthritis: insights from various rat hindlimb muscles. *Oxidative Medicine and Cellular Longevity.* 2019:1-11.

## 3.1 Abstract

Due to atrophy, muscle weakness is a common occurrence in rheumatoid arthritis (RA). The majority of human studies are conducted on the *vastus lateralis* muscle – a muscle with mixed fibre type – but little comparative data between multiple muscles in either rodent or human models are available. The current study therefore assessed both muscle ultrastructure and selected redox indicators across various muscles in a model of collagen-induced rheumatoid arthritis in female Sprague-Dawley rats. Only three muscles, the *gastrocnemius, extensor digitorum longus* (*EDL*) and *soleus*, had lower muscle mass (38%, 27% and 25% loss of muscle mass respectively; all at least $P<0.01$), while the *vastus lateralis* muscle mass was increased by 35% ($P<0.01$) in RA animals when compared to non-RA controls. However, all four muscles exhibited signs of deterioration indicative of rheumatoid cachexia. Cross-sectional area was similarly reduced in *gastrocnemius, EDL* and *soleus* (60%, 58% and 64% respectively, all $P<0.001$), but *vastus lateralis* (22% smaller, $P<0.05$) was less affected, while collagen deposition was significantly increased in muscles. This pathology was associated with significant increases in tissue levels of reactive oxygen species (ROS) in all muscles except the *vastus lateralis,* while only the *gastrocnemius* had significantly increased levels of lipid peroxidation (TBARS) and antioxidant activity (FRAP). Current data illustrates the

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differential responses of different skeletal muscles of the hindlimb to a chronic inflammatory challenge both in terms of redox changes and resistance to cachexia.

## 3.2 Introduction

There can be no doubt that individuals suffering from rheumatoid arthritis (RA) have significantly decreased quality of life. In addition to the chronic pain and other primary symptoms arising from the inflammatory processes in joints, the majority of patients also report skeletal muscle weakness (Yamada *et al.*, 2017). However, there is a disconnect between the degree of rheumatoid cachexia (defined as arthritis-associated loss of muscle mass with little or no loss of fat mass (Walsmith and Roubenoff, 2002) and the relatively more severe degree of muscle weakness experienced. Initially, the more severe loss of strength was ascribed to joint deformation and pain (Helliwell and Jackson, 1994), but more recently contractile dysfunction, mediated by tumour necrosis factor (TNF), was implicated, with TNF reported to decrease specific force by increasing cytosolic oxidant activity in the muscle (Reid and Moylan, 2011).

The pathology of skeletal muscle in patients with RA is clearly complex and much research has already been conducted in this context, so that at least a partial picture of role players is available. For example, while neuromuscular fatigue (assessed by electromyography) was reported to weakly correlate with subjective perception of fatigue and physical activity level, it did not correlate to either clinical profile or treatment features (do Espírito Santo *et al.*, 2018). This suggests that while advancement of emotional well-being should form part of treatment strategy, neuromuscular pathology is probably not a major role player in RA. In contrast, increased muscle inflammatory cytokine levels, altered expression of genes involved in muscle repair and glycolytic metabolism, as well as increased levels of fibrosis-associated amino acids, correlated with disease progression, physical inactivity and pain in a large cohort of RA patients (Huffman *et al.*, 2017). These data, generated from the *vastus lateralis* muscle, and specifically the finding related to altered glycolytic metabolism, raised the question of whether different muscles or muscles with different fibre type distribution may be differentially sensitive to RA-associated pathology.

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Given both the significant implication of inflammation in RA and the interlinked nature of inflammation and oxidative stress, attention should also be extended to redox status, in order to form a more holistic picture of pathological maladaptations that could potentially be targeted by intervention. In an elegant study on slow-twitch *soleus* muscle from rats subjected to collagen-induced arthritis (CIA), peroxynitrite-induced oxidative damage to myofibrillar proteins was implicated in measured deficits of muscle force production, such as shorter maximal contraction velocity and slower twitch contraction and relaxation (Yamada *et al.*, 2009). The same group subsequently illustrated similar deficits in force production – also ascribed to reactive nitrogen species – in fast-twitch *extensor digitorum longus* (*EDL*) muscle (Yamada *et al.*, 2015). These studies sketch a fairly fibre-type independent picture of muscle pathology in this model. However, these observations were made at a relatively advanced time point (≈day 45, with last booster shot on day 28) and does not allow for direct interpretations on specific mechanisms at play during the earlier, disease development phase. Furthermore, the degree to which different muscle types are compromised in CIA have not been comprehensively and comparatively assessed.

The aim of the current study was therefore to assess muscle morphology and selected aspects of muscle pathology and redox changes in four different hindlimb muscles in rats with collagen-induced arthritis. A somewhat milder model (day 35, with last booster shot on day 8) was employed in order to elucidate the extent to which free radical involvement and endogenous antioxidant mechanisms may contribute to early pathology in different muscles.

## 3.3 Materials and methods

### 3.3.1 Ethics statement and animal handling

The Stellenbosch University Animal Research Ethics Committee ethically cleared this study (Protocol number: SU-ACUD17-00034). Twenty (20) female Sprague-Dawley rats weighing 180 – 200 g were procured from the Stellenbosch University small laboratory animal breeding facility. They were housed in groups of 5 rats per cage in a temperature- and humidity-controlled room (23 ± 1°C, 40–60% humidity) with a set 12 h light-dark cycle and fed standard commercially available rat chow and

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tap water *ad libitum*. After acclimatization to the new environment for about 7 days, rats were randomly divided into two groups of 10 rats each - normal control (NC) and collagen-induced rheumatoid arthritis (RA). All experimental animals received humane care according to the principles outlined in the National Research Foundation Guide for Care and Use of Laboratory Animals.

### 3.3.2 Collagen-induced rheumatoid arthritis model

*Chemicals:* Bovine collagen type II, incomplete Freund's adjuvant and rat anti-collagen IgG ELISA kit were purchased from Chondrex Inc., WA, USA. Isoflurane (Isofor) was purchased from Safeline Pharmaceuticals, Johannesburg, South Africa. All other chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich (MO, USA) or Merck (Darmstadt, Germany) unless otherwise stated.

*Experimental design:* The well-established rat collagen-induced arthritis (CIA) method (Kannan, Ortmann and Kimpel, 2005; Asquith *et al.*, 2009) was used to induce arthritis in the RA group. Briefly, bovine heterologous type II collagen was first dissolved in 0.01N glacial acetic acid (2 mg/ml) before an emulsion was prepared using an equal volume of incomplete Freund's adjuvant. This emulsion was slowly injected intra-dermally twice just above the tail region of each rat under isoflurane anaesthesia, 7 days apart. The time of onset of swelling in rat paws was recorded. The progression of clinical symptoms was monitored daily and scored as follows: 0 = no swelling; 1 = erythema and digits swollen; 2 = erythema, digits and pad swollen; 3 = erythema, digits and pad swollen, joints and entire leg swollen. The total score for each rat was given as the addition of all affected paws, so the highest attainable score was 10 for each rat, as previously described (Hawkins *et al.*, 2015).

After the 5-week experimental period, all rats were killed by guillotine decapitation and the trunk blood was immediately collected into tubes *via* a heparinized funnel. The plasma was subsequently separated after centrifugation at 2,000 *g* for 10 minutes using a Spectrafuge 24D centrifuge (Labnet International Inc., NJ, USA). *EDL*, *gastrocnemius*, and *soleus* and *vastus lateralis* muscles from the hindquarters of each rat were removed carefully, weighed, snap-frozen in liquid nitrogen and then stored at -80 $^{o}$C until subsequent analysis.

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*Validation of model via plasma anti-collagen IgG titer:* The successful induction of arthritis in rats was confirmed by the production of antibodies to type II collagen using the rat anti-collagen IgG ELISA kit (Chondrex Inc., WA, USA) and following the manufacturer's protocol.

### 3.3.3 Muscle histology

Frozen tissues (*EDL*, *gastrocnemius, soleus* and *vastus lateralis*) were sectioned in 10 µm cross-sections with a cryostat (Leica CM1860 UV, Leica Biosystems Nussloch GmbH, Germany) at -25°C and stored at -20°C. To ensure consistency between samples a predetermined section was cut off the proximal end of each sample (differed between muscle types) before sectioning occurred. This is particularly important in the *vastus lateralis* as different fibre type proportions exist in the different areas (superficial vs. deep and proximal vs. distal) (Kohn and Myburgh, 2007). Sections were allowed to thaw at room temperature for 20 minutes before staining.

*Hematoxylin & Eosin staining*: H&E staining was used to view the overall muscle structure. Hematoxylin binds to and stains all DNA/RNA structures blue. Eosin counterstains all proteins of the tissue pink. Slides were submerged in the following order for one minute each, excluding Eosin which was stained for 30 seconds: $dH_2O$, two changes of Mayer's haematoxylin, warm tap water, Scott's tap water, $dH_2O$, Eosin, $H_2O$, 95% Ethanol, 100% Ethanol, and finally clearance in xylene. Once stained, the slides were mounted with mountant media (DPX, 06522, Sigma-Aldrich, USA) and covered with a cover-slip for viewing.

*Picrosirius red staining:* Sirius red is a polyazo dye which is specifically used for staining collagen. The stain dyes collagen bright red, leaving muscle fibres, cytoplasm and red blood cells a lighter yellow/red colour. Picrosirius red differs from Sirius red staining with the addition of picric acid which prevents the indiscriminate staining of non-collagenous structures by Sirius red.

Sections were fixed in Neutral Buffered Formalin for 30 minutes. Slides were rinsed in $dH_2O$ and stained with Weigert's Haematoxylin for 8 minutes. Sections were washed in 3 changes of water followed by staining with Picrosirius solution for one hour. Picrosirius solution was made up of 0.5 g Sirius Red F3B (CI35780, Sigma-

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Aldrich, USA) in 500 ml saturated aqueous Picric Acid (197378, Sigma-Aldrich, USA). Sections were washed in 0.5% acetic acid (A6283, Sigma-Aldrich, USA) in $dH_2O$ twice for 5 minutes each. Sections were then dehydrated in changes of ethanol (70%, 95%, 100%), and then cleared in xylene (296325, Sigma-Aldrich, USA) and cover slips were mounted with mountant media (DPX, 06522, Sigma-Aldrich, USA).

*Image acquisition*: All histological slides were viewed using bright-field microscopy (Nikon ECLIPSE E400), mounted with a camera (Nikon DS-Fi2), and processed through a Digital Sight processor (DS-U3, Nikon, Japan). Image processing was done on Nikon Instruments Software (NIS-Elements v4.10) on a desktop computer (Dell, USA) running Windows 7 (Microsoft, USA). Images were taken at 100x and 200x magnification. (Magnification was calculated from ocular lens (10x) multiplied by objective lens (10x/20x)).

*Image analysis*: The cross-sectional area of the fibres was measured using H&E sections and Image J software (version 1.49, Wayne Rasband). 50 fibres per sample were measured. In order to assess fibrosis, picrosirius red images were analysed using Image J software (version 1.49, Wayne Rasband) with the Colour Deconvolution plug-in as developed by Landini (version 1.5). The picrosirius stains were processed using the Image J RGB option. Briefly, the plug-in unmixes the RGB image into three 8-bit images with a colour look up table that corresponds to the respective vector colours. The analysis measurements were set to measure ‘area’, ‘area fraction’, ‘limit to threshold’ and ‘display label’. The threshold of each of the three images was adjusted allowing the measurement of 1) the background, 2) the connective tissue, and 3) total tissue. Percentage of fibrosis in the tissue (x100 magnification) was calculated as follows: 1) background was subtracted from both the connective tissue and total tissue; 2) the following formula was used: Percentage of fibrosis = (connective tissue/(connective tissue + total tissue))*100.

### 3.3.4 Sample analysis for redox status

*Oxidative stress:* Frozen *vastus lateralis, soleus, EDL* and *gastrocnemius* tissues were thawed on ice and homogenized 100 mg/ml in 10 mM phosphate buffered saline (PBS, pH 7.2) and centrifuged at 10,000 *g* for 15 min at 4 $^{o}$C to obtain the supernatants used for the analyses. The presence of reactive oxygen species (ROS)

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in tissue homogenates was evaluated by the ROS-dependent oxidation of the non-fluorescent 2', 7', -dichlorofluorescein (DCF)-DiOxyQ probe to the highly fluorescent DCF using the OxiSelect$^{TM}$ ROS assay kit (Cell Biolabs, Inc, CA, USA) and following the manufacturer procedure. The ferric ion reducing antioxidant power (FRAP) value in all muscle homogenates was determined by measuring the reduction of ferric tripyridyltriazine ($Fe^{3+}$-TPTZ) complex to the ferrous ($Fe^{2+}$) form by antioxidants as detailed earlier by. This is monitored by the change in absorption at 593 nm in a SPECTROstar Nano® absorbance plate reader (BMG Labtech, Ortenberg, Germany). Lipid peroxidation in muscle homogenates was evaluated by the formation of the stable product – malondialdehyde (MDA) – in a reaction medium containing thiobarbituric acid (TBA). We used the method described by but with slight modifications: briefly, the MDA formed in tissues highly reacts with TBA under acidic conditions to form a complex that is better purified by the addition of butanol and saturated sodium chloride and absorbs maximally at 532 nm using a SPECTROstar Nano® absorbance plate reader (BMG Labtech, Ortenberg, Germany).

### 3.3.5 Statistical analysis

Effects were compared for statistical significance using student t-tests, or one- or two-way analysis of variance (ANOVA) as appropriate, with Bonferroni post hoc tests where applicable. Data are presented as means and standard deviations, unless otherwise indicated. Statistical significance were set at $P<0.05$.

## 3.4 Results

Successful induction of relatively mild rheumatoid arthritis was confirmed by clinical observations (Figure 3.1). Onset of joint swelling was typically within 11-18 days after the initial exposure to collagen. In addition, significantly elevated levels of anti-collagen antibody titre were measured in RA animals (0.02 ± 0.007 g/ml plasma; $P<0.0001$) when compared to non-RA controls, in which antibody levels were not detectable.

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RA animals exhibited significant decreases in skeletal muscle mass in the EDL, *gastrocnemius*, and *soleus* muscles, but that of *vastus lateralis* significantly increased (Figure 3.2).

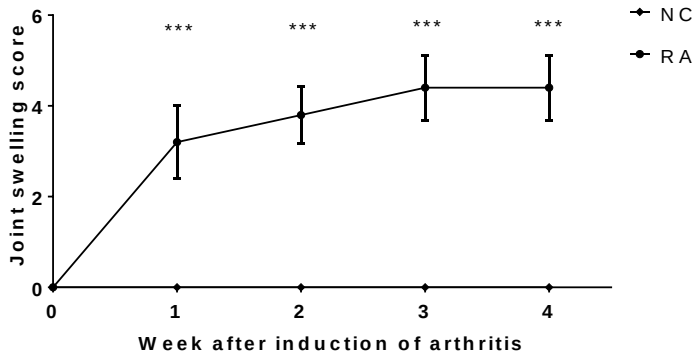


*Figure 3.1: Weekly scores indicative of clinical symptoms of arthritis development in a collagen-induced rheumatoid arthritis model in female Sprague-Dawley rats (n=10 per group). Statistical analysis: one-way ANOVA with repeated measures and Bonferroni post hoc testing. ***, p<0.0001 significantly different from controls.*

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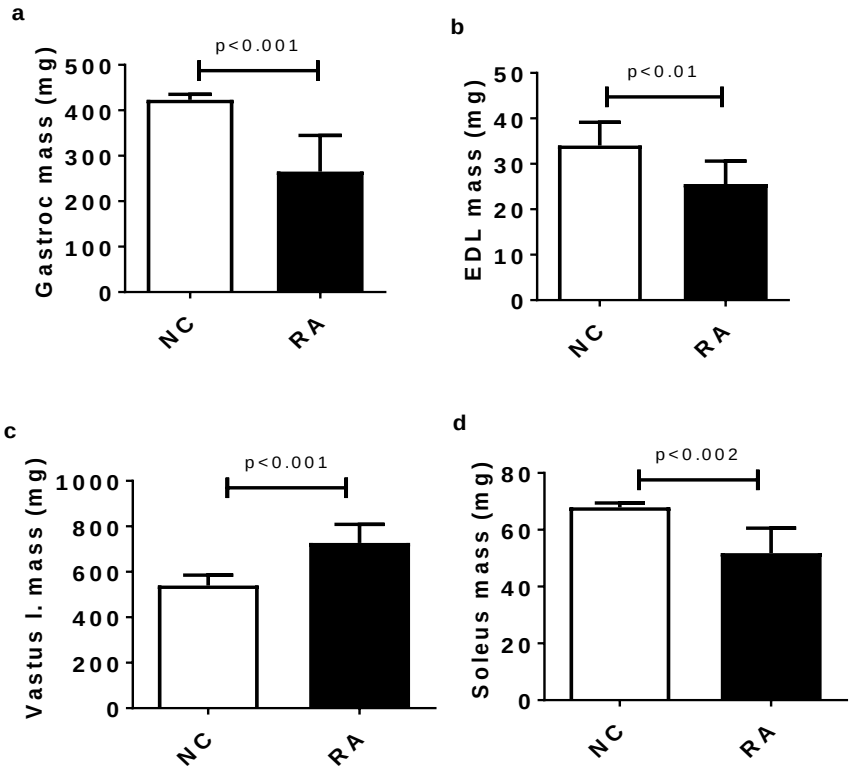


*Figure 3.2: Muscle mass of different rat hindlimb skeletal muscles on day 35 of CIA (n=10 per group). Bars indicate mean mass of muscle (left and right muscle weights were averaged for each animal) and error bars are standard deviations. Statistical analysis: Student t-test.*

RA animals exhibited clear signs of myofibre atrophy (Figure 3.3 and 3.4), inflammation (Figure 3.5) and fibrosis (Figure 3.6) when compared to control animals. In line with the significantly decreased muscle mass, *gastrocnemius, soleus* and *EDL* muscle exhibited generalised cachexia which was characterised by a ≈60% reduction in myofibre cross-sectional area across all cells (Figure 3.3a-f, j-l). In these muscles, a significant number of fibres undergoing degradation were visible, as well as oedema and inflammatory cell infiltrate. In contrast, as suggested by the lack of muscle mass loss, *vastus lateralis* myofibres seemed least affected by RA, with cross-sectional area of fibres decreasing by only ≈20% (Figure 3.3g-i). In this muscle group, the pattern of cachexia was also more varied: while some fibres of smaller cross-sectional area is visible, normal sized fibres are still abundant. No evidence of myofibre degradation was visible in *vastus lateralis* sections analysed.

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An analysis of fibre size distribution (Figure 3.4) confirms these observational data. *Gastrocnemius, soleus* and *EDL* muscle exhibited a shift to the left for fibre size, with very high frequency of small fibre size. The *vastus lateralis* muscle did not show a clear shift to the left, although frequency of smaller fibres did appear somewhat higher. Although inflammation was not a specific focus of this paper and specific inflammatory markers were not assessed, the RA animals clearly exhibited moderately severe levels of inflammation in the *soleus, gastrocnemius* and *EDL* muscles, where inflammation was visible in the perivascular areas as well as in between individual myofibres (Figure 3.5 a-b). In addition, intrafibre necrosis was visible in several cells (Figure 3.5c). These features were not clearly visible in *vastus* muscle.

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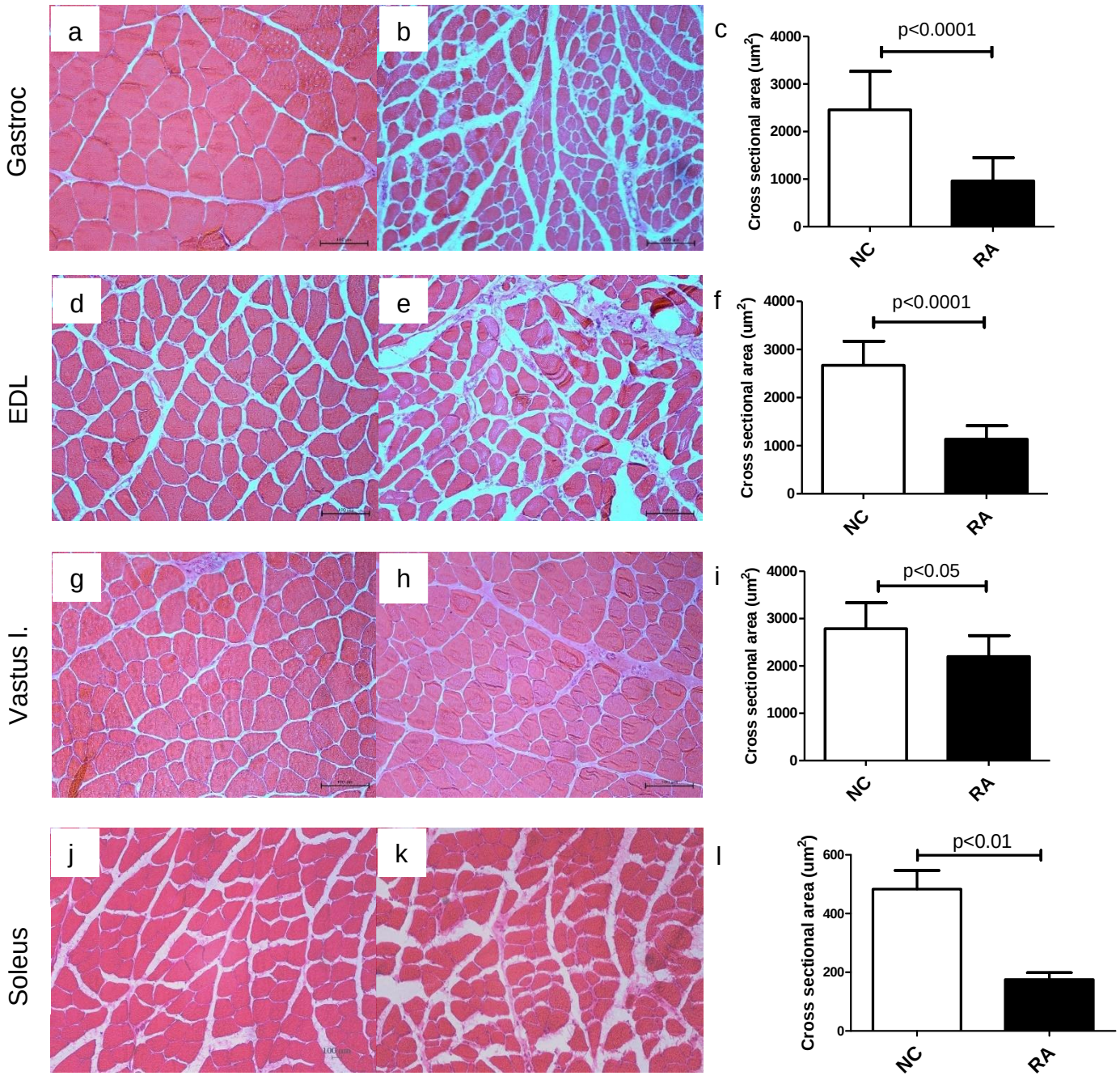


*Figure 3.3: Representative H&E images and cross-sectional area of gastrocnemius (a-c), EDL (d-f) and vastus lateralis (g-i) muscle depicting ultrastructural changes in female rats subjected to CIA (n=10 per group) (normal control left; CIA right). 200x magnification. Scale bar represents 100 µm. Statistical analysis: Student t-test*

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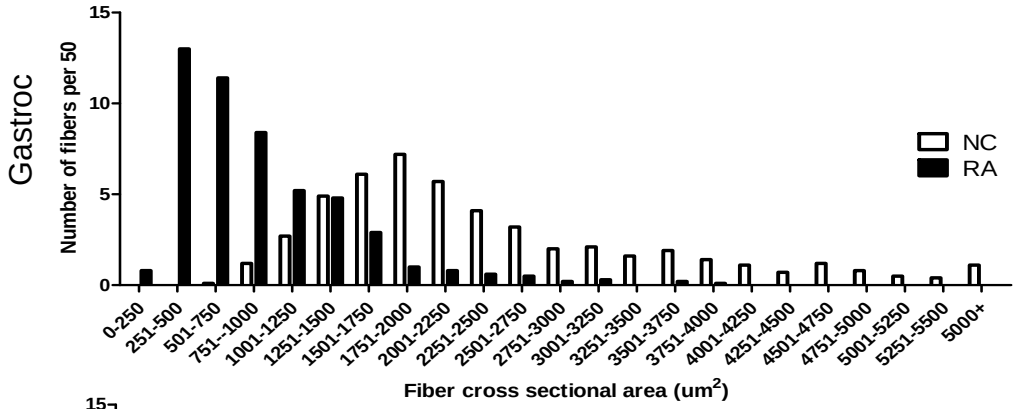
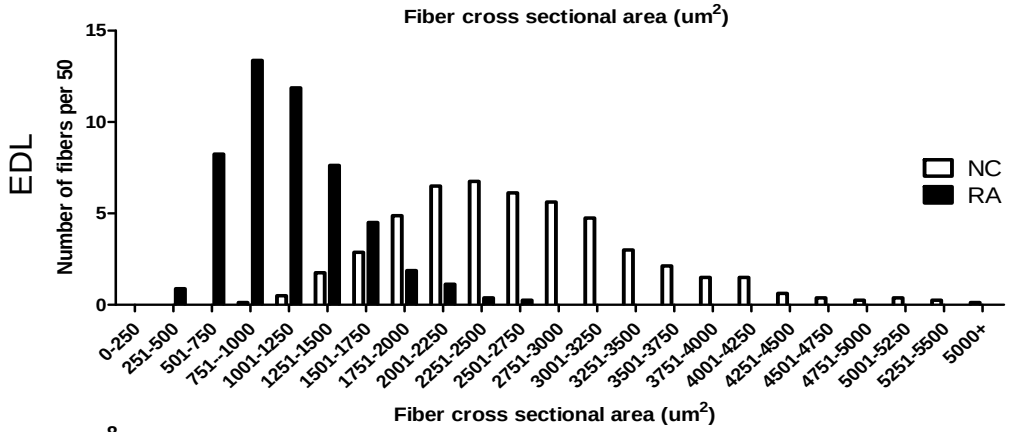
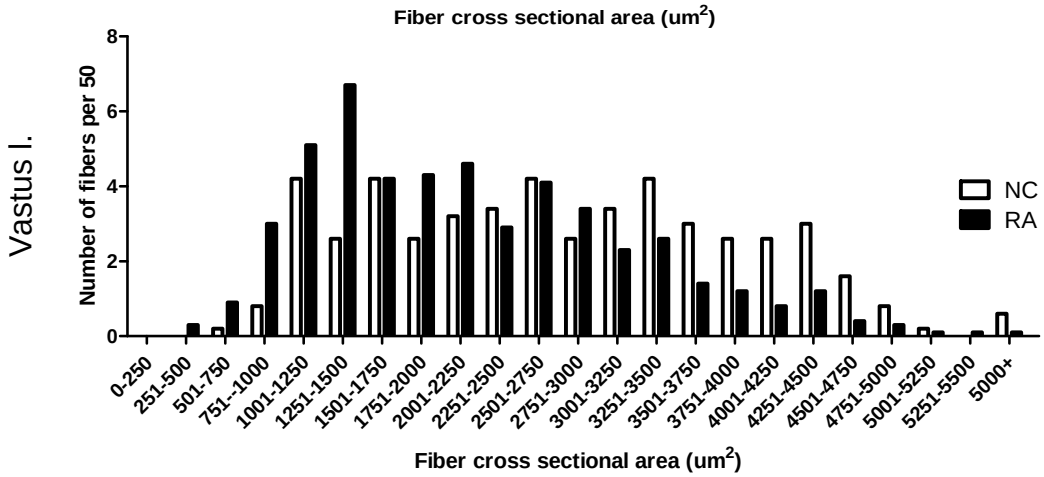
a



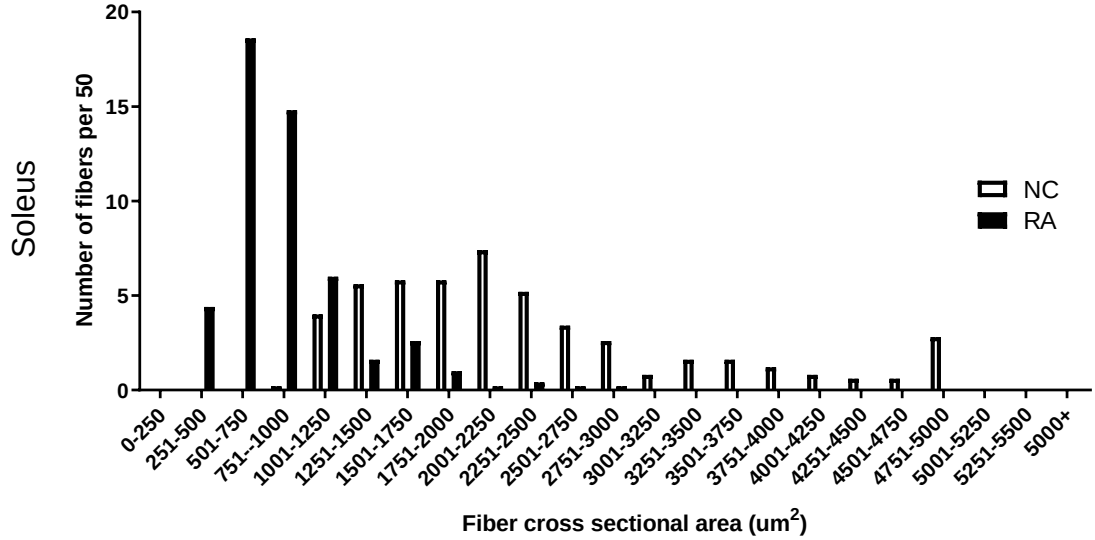

b




c




d



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*Fiigure 3.4: Distribution of fibre cross-sectional area across different muscle groups (n=10 per group). Frequency data is expressed as number of fibres out of a total of 50 fibres counted per sample.*

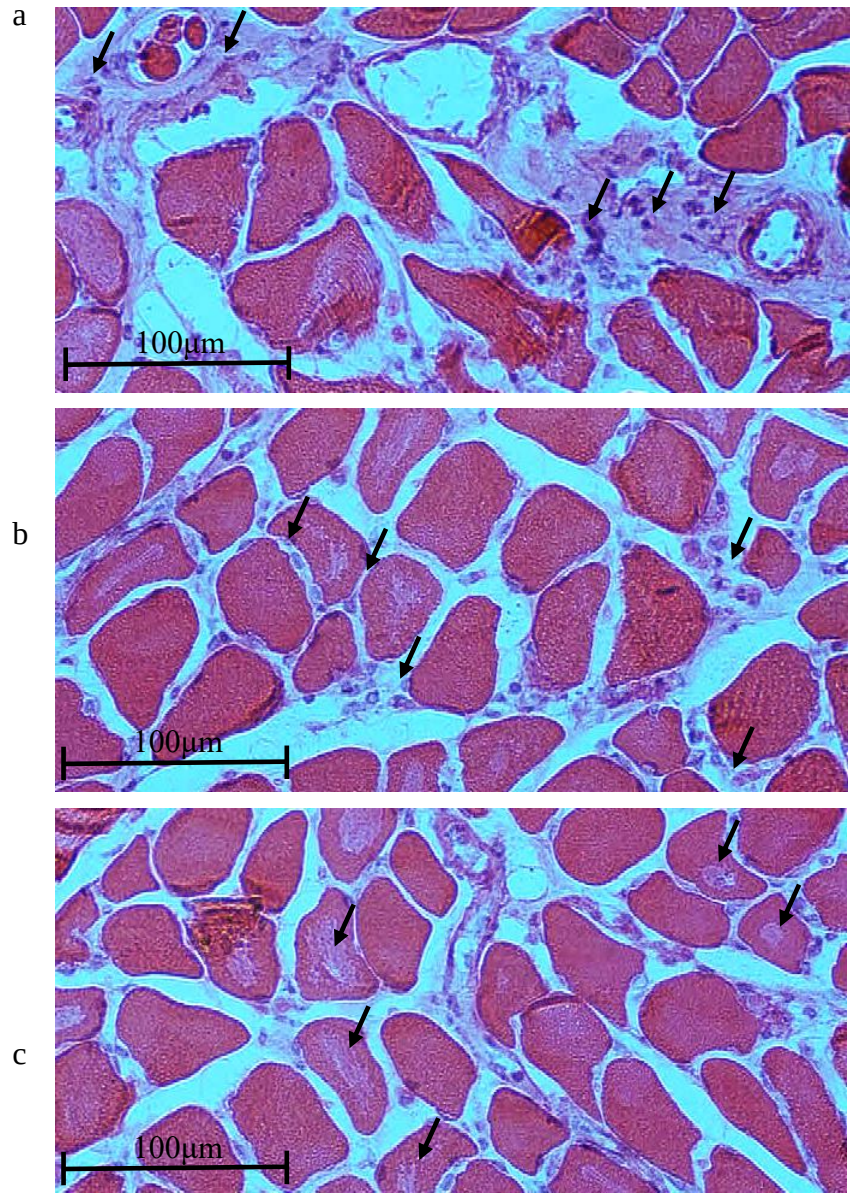


*Figure 3.5: Representative high-resolution H&E stained images illustrating inflammatory cell infiltration in (a) perivascular and (b) interfibre areas, as well as (c) intrafibre necrosis. 200x magnification. Scale bar represents 100µm.*

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In terms of fibrosis (collagen accumulation), the *vastus lateralis* and *EDL* muscles exhibited a significant 50% increase when compared to their respective controls. A similar result was obtained in the *soleus* but did not reach statistical significance. In contrast, the *gastrocnemius* muscle was most severely compromised, exhibiting a striking 200% increase in collagen accumulation (Figure 3.6).

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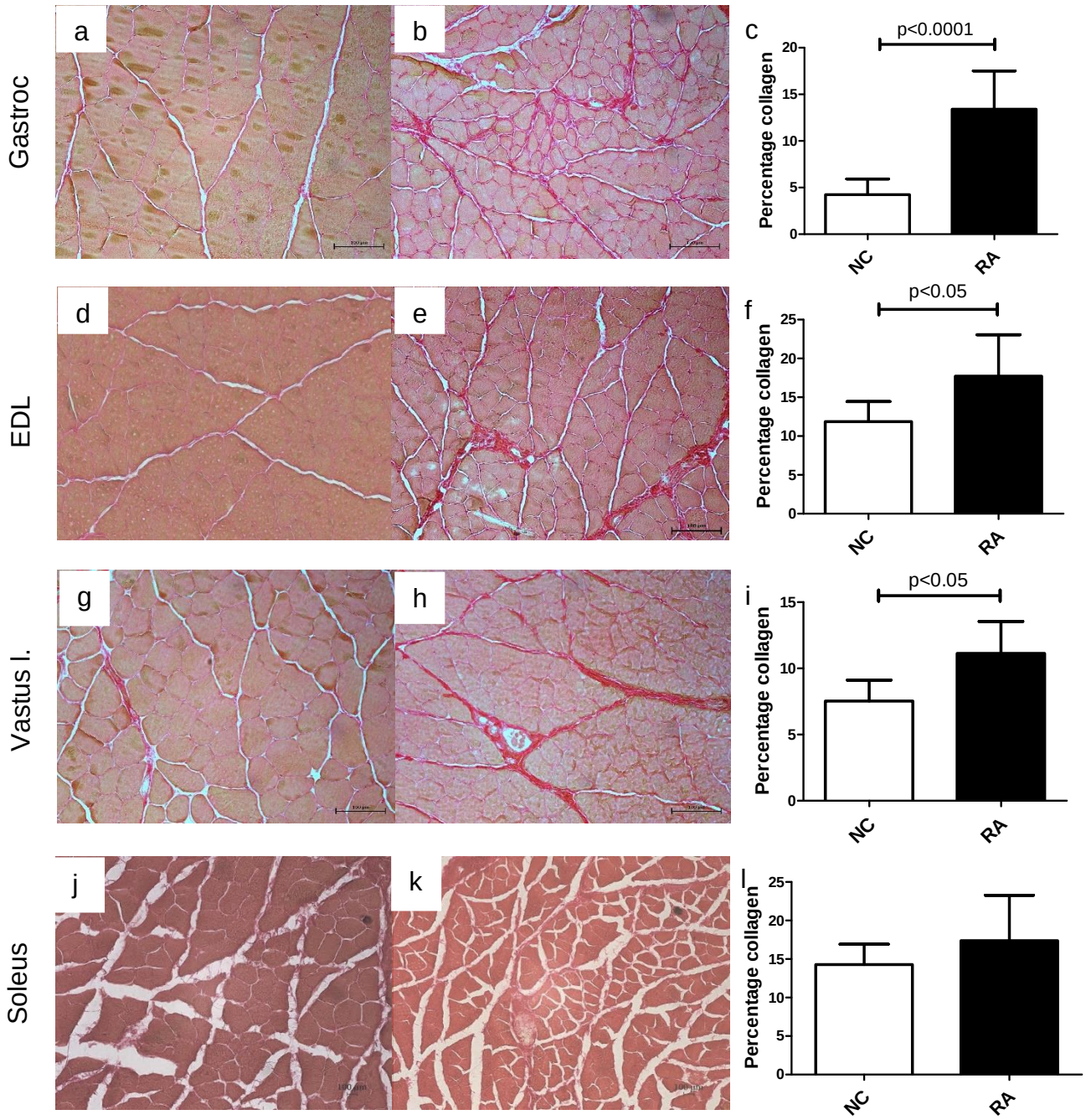

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*Figure 3.6: Representative images and percentage fibrosis of gastrocnemius (a-c), EDL (d-f) and vastus lateralis (g-i) in female rats subjected to CIA (n=10 per group) using Picrosirius red staining (normal control left; CIA right). 200x magnification. Scale bar represents 100 µm. Statistical analysis: Student t-test*

In terms of redox status, total reactive oxygen species (ROS) levels seemed to correspond to muscle pathology in RA, as ROS was significantly increased in *gastrocnemius, EDL* and *soleus*, but not *vastus,* muscle of RA animals when compared to controls (Figure 3.7a). TBARS was assessed as measure of oxidative stress-associated membrane damage through lipid peroxidation (Figure 3.7b). When considering controls only, the lowest ROS production seen in the *gastrocnemius* muscle corresponded to lowest levels of TBARS in this muscle, with highest TBARS levels were measured in the *EDL* and *soleus* muscle. This relatively poorer picture in terms of oxidative damage in control *EDL* and *soleus* muscle was not matched by a relative increase in antioxidant capacity (FRAP) (Figure 3.7c), suggesting that under normal conditions, these muscles are relatively more compromised than the *gastrocnemius* muscle in this context. In RA animals, lipid peroxidation (TBARS) levels were only significantly elevated from control levels in the *gastrocnemius* muscle (Figure 3.7b). Similarly, antioxidant capacity (FRAP) was similar and unchanged by RA in most muscles, with the exception of the *gastrocnemius* muscle, in which it was significantly increased in response to RA (Figure 3.7c).

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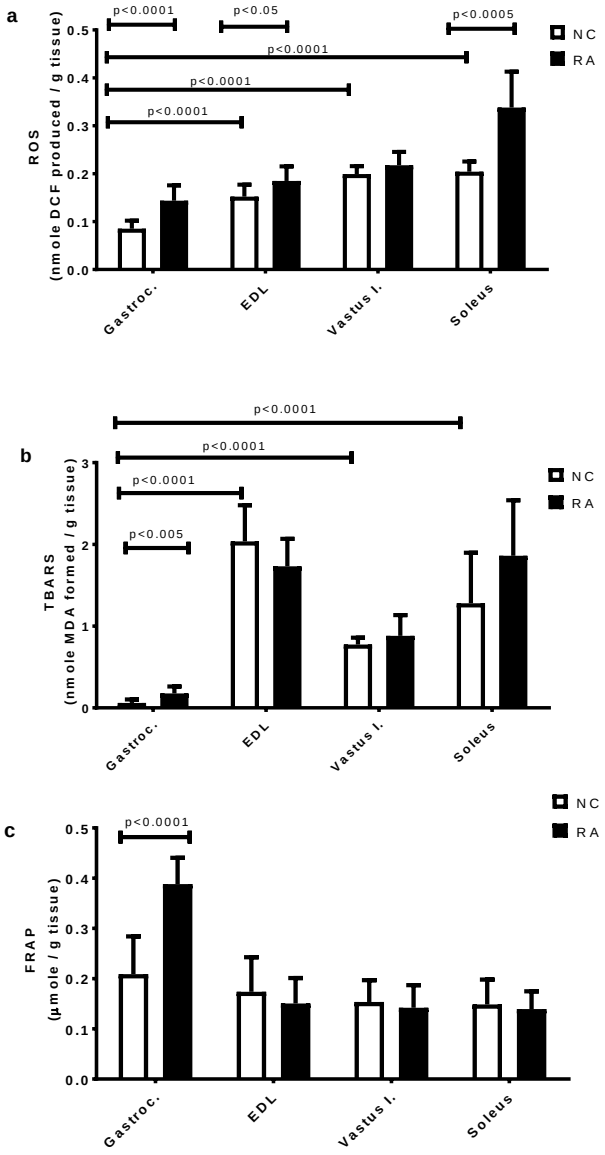


*Figure 3.7: Redox status of rat muscles in female rats subjected to CIA, as measured by total ROS (a), TBARS (b) and FRAP (c) assays respectively (n=10 per group). Bars indicate means and error bars are standard deviations. Statistical analysis: 2-way ANOVA with Bonferroni post hoc tests*

## 3.5 Discussion

The current study expands on available literature by presenting a comparative assessment of different hindlimb skeletal muscles affected by CIA in a rat model. A specific novel component is the comparison of four different muscles with different fibre type distribution, including the *vastus lateralis,* in which human studies are

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typically conducted. Importantly, a recent study conducted in humans reported that intramuscular (*vastus lateralis*) levels of inflammatory parameters, such as the inflammatory cytokines, did not correspond to the profile in circulation (Huffman *et al.*, 2017), which highlights the importance of tissue-specific investigation and the relevance of the study reported here.

Data presented here clearly illustrates the debilitating nature of CIA and validates this model as an accurate simulation of muscle pathology in rheumatoid arthritis. Current data for the first time provides a comparative histological assessment of four muscles, three of which (*gastrocnemius, soleus* and *EDL* muscles) are directly associated with the primarily affected joint (ankle), while the other (*vastus lateralis*) is more proximal. The fact that all four muscle groups exhibited signs of cachexia, albeit it much less severe in the *vastus lateralis*, suggests that inactivity-based atrophy is not the only contributor to the pathology observed, and that circulating mediators likely impact significantly on all muscle groups, irrespective of their anatomical position. This generalised muscle deterioration is in line with the human clinical profile of RA (Sokka *et al.*, 2008) and further validates our model.

The smaller fibre size observed in all muscle groups assessed in the current study, could theoretically be the result of a shift in fibre type favouring type I oxidative fibre phenotype, which is generally smaller than glycolytic myofibres. This is however unlikely, since skeletal muscle wasting in COPD and other chronic diseases characterised by chronic inflammation, has been reported to be associated with a shift in phenotype towards type II glycolytic fibres (Yu *et al.*, 2008; Remels *et al.*, 2013) – which are incidentally also most susceptible to cachexia in general (Minnaard *et al.*, 2005; Li *et al.*, 2007) and specifically also to arthritis-associated cachexia (López-Menduiña *et al.*, 2010). This led to the hypothesis that a loss of muscle oxidative phenotype may result in increased susceptibility to inflammation (Remels *et al.*, 2013) and oxidative stress-induced cachexia.

The inclusion of more muscle groups in the current study provided the opportunity to gain novel insights and indeed suggested that fibre type and metabolic preference alone are not major determinants of the sensitivity of myofibres to rheumatoid cachexia. In the current study, the extent of muscle fibre atrophy was similar for the *soleus, gastrocnemius* and *EDL* muscles – muscles which share an anatomical site

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in the lower hindlimb but differ in terms of fibre type distribution and metabolic preference. The lack of more extended time points in the cross-sectional design of the current study however did not allow for assessment of change within the same muscle over time, to determine if muscles with different fibre type or metabolic preference may respond differently over time or with disease progression. Thus, causal mechanisms potentially at play should be investigated in a study with longitudinal design including early, medium and long-term disease progression time frames. The extent of inflammation and its effect on muscle deterioration should also be considered in such a study of longitudinal design.

In the current study, diffusely spread inflammation was clearly visible in muscle assessed, but a comprehensive assessment of inflammation was beyond the current scope. Nevertheless, skeletal muscle fibrosis has previously been linked to inflammatory processes and was quantitatively assessed. Fibrosis resulting from inflammation and associated oxidative damage in various models specifically links ROS production to modulation of transforming growth factor beta (TGF-β) signalling in the context of mitochondrial dysfunction (Kozakowska and Alicja, 2015; Liu and Desai, 2015; Gonzalez-Gonzalez *et al.*, 2017; Maezawa *et al.*, 2017). This prompted an investigation into potential differences in redox status between the different muscles. Indeed, in the current study, even in the absence of CIA, *vastus lateralis, EDL* and *soleus* muscles exhibited higher levels of TBARS than the *gastrocnemius* muscle, which also exhibited lower ROS levels than all other muscles assessed. This is in line with previous reports of differential inherent superoxide dismutase activity, glutathione peroxidase activity and malondialdehyde levels in different muscles (diaphragm, *soleus* and *gastrocnemius*) (Caillaud *et al.*, 1999). When subjected to CIA, even though ROS increased in the *gastrocnemius, EDL* and *soleus*, FRAP remained unchanged in the *EDL* and *soleus*, while in contrast, in *gastrocnemius* muscle, FRAP increased significantly in response to the increased ROS of RA. Together, these data suggest that the *EDL* and *soleus* muscles may have relatively less inherent antioxidant capacity to react to oxidative stressors when compared to the *gastrocnemius* muscle, which was able to mount a significant antioxidant response. Despite this, the *gastrocnemius* muscle exhibited a worse profile in terms of malondialdehyde (MDA) production. We propose that the *EDL* and *soleus* muscles may possess antioxidant mechanisms other than those assessed by the

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FRAP assay, which could have upregulated activity in response to CIA and by which membrane integrity may be maintained. These mechanisms would be independent of ferric iron reduction specifically, which is assessed by the FRAP assay reported here. This interpretation is in line with the report in a model of high-fat diet-induced oxidative stress in rats, which reported that increased ROS production (reduced mitochondrial $H_2O_2$ emission, increased palmitate oxidation and increased mRNA expression of NADPH complex) in muscle was paralleled by an increased ROS buffering capacity (increased mRNA expression of the antioxidant proteins manganese superoxide dismutase (MnSOD), glutathione reductase and mitochondrial thioredoxin-dependent peroxide reductase 3 and 5) (Pinho *et al.*, 2017). Another mechanism by which this may potentially be achieved in the current context, is that *EDL* and *soleus* muscles may contain relatively higher concentrations of Vitamin E, which is known to contribute to cell membrane repair after oxidative damage. In line with this, although the majority of data was generated in pre-clinical models and no comparison between muscle with different fibre types could be found, several beneficial effects of Vitamin E were recently reported in the context of age-related sarcopenia. These included myoblast proliferation and differentiation, survival, membrane repair, mitochondrial efficiency and maintenance of muscle mass and contractile capacity (Chung *et al.*, 2018). Of particular relevance to the current discussion, vitamin E supplementation were shown to prevent upregulation of muscle ring finger 1 (MuRF1) and caspase-9 and -12 mRNA in unloaded rat muscle and to decrease upregulation of muscle calpain, caspase-3 and atrogin-1 (Mafbx) mRNA (Servais *et al.*, 2007). This would suggest that in addition to its antioxidant effect, vitamin E may also modulate atrophy via more direct inhibitory action on proteolytic pathways. The review by Chung and colleagues (2018) highlighted the lack of clinically relevant information currently available on vitamin E content or protective properties in skeletal muscle, which up to now has been limited by methodological constraints. This warrants further investigations in order to elucidate how endogenous protective mechanisms may be exploited by exogenous means for therapeutic benefit.

Redox status is a major determinant of muscle pathology – and specifically chronic disease-related cachexia. The current study focussed on oxidative stress resulting from reactive oxygen species. However, both oxidative and nitrosative stress may

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also come into play here, as both have been implicated in cancer-cachexia (Barreiro *et al.*, 2005). In addition, in rodents injected with lipopolysaccharide (LPS) to induce oxidative stress, inducible nitric oxide synthase (iNOS) was implicated as role player in relatively greater resistance to atrophy and ubiquitin-associated protein degradation in oxidative, but not glycolytic fibres (Yu *et al.*, 2008). This suggests that future studies should also determine the interaction between these species but also that antioxidant treatments should be investigated as preventative modality or adjuvant treatment in RA. Indeed, astaxanthin – a powerful antioxidant – were reported to attenuate immobilisation-induced increased collagen deposition via modulation of oxidative stress, most notably via altered SOD-1 and TGF-β signalling in rats (Maezawa *et al.*, 2017). Similarly, the antioxidant flavone mixture isoflavin-β was reported to attenuate toxin-induced loss of *gastrocnemius* muscle mass by preventing the oxidative modification of proteins (Marinello *et al.*, 2018).

Another observation that has remained unnoticed up to now, is the fact that in the rodent model of CIA, the response in the *vastus lateralis* muscle was different from other muscles assessed here, and also different from results previously reported for the *EDL*, and *soleus* in rodent models of arthritis (Yamada *et al.*, 2009, 2015). Current data suggest that although the *vastus lateralis* myofibres exhibited some signs of deterioration, total muscle mass actually increased significantly. The fibre size variation in this muscle did not display the characteristic left shift associated with cachectic atrophy, although the mean cross-sectional area was somewhat reduced. This suggests that the *vastus* may have been recruited as usual for gait despite the presence of RA-related symptoms. In contrast, the severe pathology evident in the *gastrocnemius* and *EDL* muscle groups may have resulted not only from systemic effects but also due to lesser recruitment for gait and posture. This result stresses the importance of data interpretation in conjunction with clinical observations.

In terms of limitations, although not directly assessed, in our opinion muscle mass is unlikely to have been influenced by energy intake, as animals did not exhibit clear loss of appetite. In terms of disuse as confounding factor, although RA animals walked with a limp due to affected joints, they still moved around readily in their cages. Furthermore, a comparative study between immobilisation and CIA-associated muscle atrophy has reported both proteolytic and regenerative pathways to be upregulated in RA (but not in experimentally immobilised) animals (de Oliveira

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Nunes Teixeira *et al.*, 2013). Thus, disuse is unlikely to have been a major confounding factor in the current study. Finally, oedema was not quantified in the current study, but may aid in the interpretation of e.g. the increased *vastus lateralis* muscle mass despite presence of other signs of atrophy. Inclusion of this measure in studies investigating inflammatory mechanisms in the context of RA-associated muscle deterioration, could further elucidate differences in the responses of different muscles to the challenges of RA.

### 3.6 Conclusion

Data presented here highlight the relevance of muscle -specific (and not just fibre-type-specific) assessment of redox profile to determine its role in muscles' atrophy response to experimentally induced rheumatoid arthritis. Furthermore, current data indicating differential responses by different muscles suggest that in human studies, it would be prudent to investigate muscle response to RA in more than one muscle. This would provide more insight into whether loadbearing and non-loadbearing muscles, or muscles at different anatomical proximities from clinically affected joints, are also differentially affected in humans in terms of disease-related cachexia. Similarly, given the inherently different redox profiles and seemingly different predominating endogenous antioxidant mechanisms at play in the different muscles as demonstrated in rats here, interventions aimed at improving redox status should take into consideration conditions in (and responses of) more muscles than just the human *vastus lateralis,* as different muscles may have different therapeutic requirements in this context.

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# Chapter 4

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## 4.1 Abstract

Rheumatoid arthritis targets numerous organs in patients, including the skeletal muscle, resulting in rheumatoid cachexia. In the muscle niche, satellite cells, macrophages and myofibroblasts may be affected and the factors they release altered. This study aimed to assess these cell types, cytokines and growth factors and their relationships to muscle fibre size and number in a rodent collagen-induced arthritis (CIA) model, in order to identify new therapeutic targets. Fibre cross-sectional area (CSA) was 57% lower in CIA than controls ($p<0.0001$), thus smaller but more fibres visible per field of view. Immunostaining indicated increased presence of satellite cells, macrophages, myofibroblasts and myonuclei per field of view in CIA ($p<0.01$), but this finding was not maintained when taking fibre number into consideration. Western blots of *gastrocnemius* samples indicated that tumor necrosis factor-α was significantly elevated ($p<0.01$) while interleukin-10 (IL-10) was decreased ($p<0.05$) in CIA. This effect was maintained (and heightened for IL-10) when expressed per fibre number. Myogenic regulatory factors (MyoD and myogenin), transforming growth factor-β and inhibitor of differentiation were significantly elevated in CIA muscle and levels correlated significantly with CSA. Several of these factors remained elevated, but bone morphogenetic protein-7 decreased when considering fibre number per area. In conclusion, CIA-muscle demonstrated good regenerative response. Myoblast numbers per fibre were not elevated, suggesting their activity results from the persistent inflammatory signalling which also significantly hampered maintenance of muscle fibre size. A clearer picture

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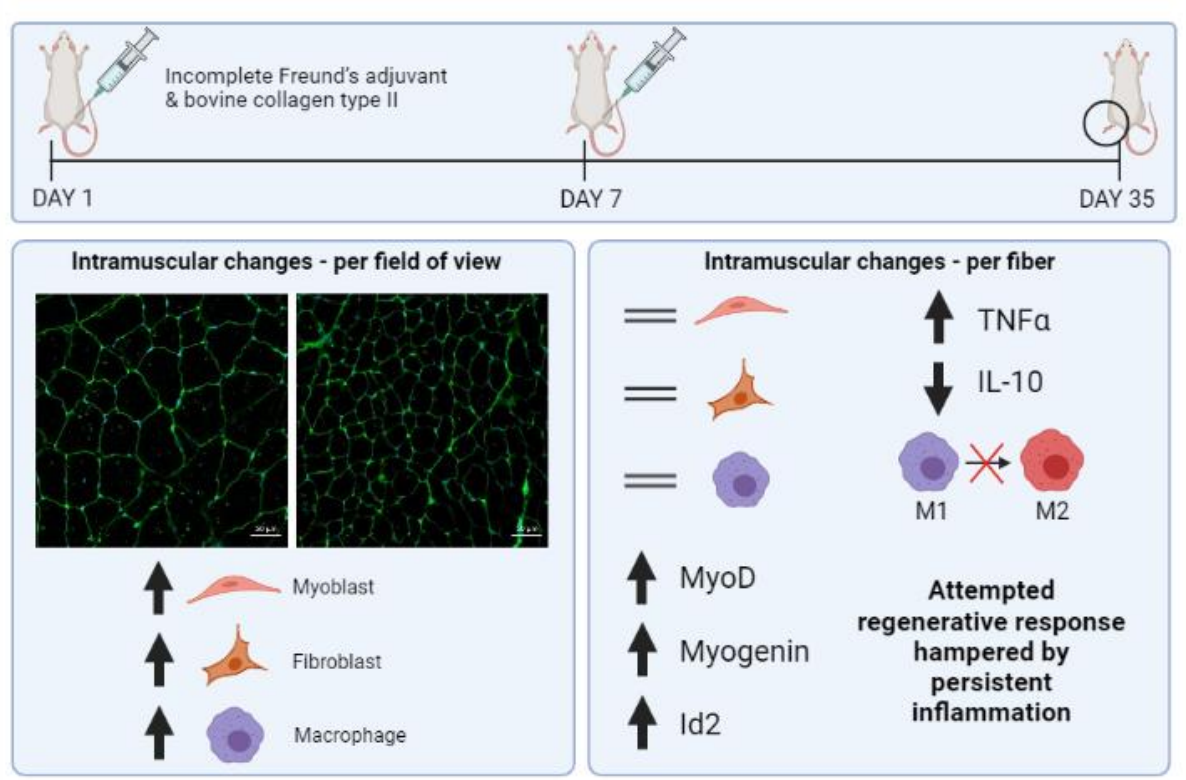
of signalling events at cellular level in arthritis muscle may be derived from expressing data per fibre.




## 4.2 Introduction

Rheumatoid arthritis (RA), an inflammatory auto-immune disease, is not limited to the synovial lining of affected joints. Despite the aetiology of RA still requiring elucidation, the disease trigger(s) is known to stimulate multiple cell types, including monocytes, fibroblasts, and T-cells, as well as the cytokines secreted by these cells, to induce and maintain the immune reaction responsible for the development of RA (Gaffo, Saag and Curtis, 2006). The chronic inflammation characterising this condition affects various organs and tissues, including skeletal muscle, resulting in substantial morbidity and increased risk of premature mortality (Mcinnes and Schett, 2011). Addressing these secondary symptoms, such as rheumatoid cachexia (Walsmith and Roubenoff, 2002; Masuko, 2014), may increase standard of living of these patients significantly. However, the limitations of clinical routine invasive assessments of RA patients is an obstacle in elucidating the full pathology at tissue level, warranting investigation using rodent models.

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In terms of pro-inflammatory signalling, tumor necrosis factor (TNF)-α is one of the key cytokines implicated in the development of RA - its presence in RA joint tissue (Chu *et al.*, 1991) and its potential to degrade cartilage is well-established (Dayer, Beutler and Cerami, 1985; Araki and Mimura, 2016). Overall, pro-inflammatory cytokines are elevated in both plasma and synovial fluid of RA patients. However, the synovial fluid exhibits greater accumulation of cytokines than blood plasma (Wright *et al.*, 2012). Similarly, plasma cytokine profile also differs from that reported from muscle biopsies of RA patients (Huffman *et al.*, 2017). Together, this suggests that the blood cytokine profile may only represent the clearance of excess cytokines secreted, rather than the actual picture at tissue level. Although there is a paucity of information, muscle inflammatory markers have been associated with disease activity, disability and pain (Huffman *et al.*, 2017), highlighting their importance in disease progression and the necessity to understand cytokine signalling at the tissue level.

One of the proposed mechanisms of rheumatoid cachexia itself is the lingering pro-inflammatory state observed in RA (Walsmith and Roubenoff, 2002). For example, in rodent collagen-induced arthritis (CIA) models, blocking TNF-α and/or IL-1β reduced the extent of muscle wasting (Roubenoff *et al.*, 1997; Dayer, 2002), while heightened pro-inflammatory and reduced anti-inflammatory cytokines were reported to interfere with regulated satellite cell activation and differentiation, resulting in reduced regenerative capacity (Teixeira, Filippin and Xavier, 2012; Ollewagen, Myburgh, *et al.*, 2021). Furthermore, chronic inflammation also dysregulates the proteins of the ubiquitin-proteasome pathway, further contributing to muscle wasting (Bodine and Baehr, 2014; Gómez-SanMiguel *et al.*, 2016), related to the unresolved inflammation. Fibrosis is a common characteristic of RA and fibroblasts are fairly abundant in all tissues (Wynn, 2019), but specifically also skeletal muscle (Agrawal *et al.*, 2003), with a significant amount of fibrosis detected in rodent CIA skeletal muscle (Oyenihi *et al.*, 2019). However, very few investigations consider the potential role of fibroblast signalling to dysregulated muscle mass maintenance in RA, and almost no data is available from a realistic simulation of the human *in vivo* scenario (Ollewagen, Myburgh, *et al.*, 2021).

Lastly, the severity and rate of progression of RA and rheumatoid cachexia, as well as individual responses to treatment, are extremely variable (Rafaela C E Santo *et*

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*al.*, 2018). This complexity of the topic may, at least in part, explain the relative lack of data regarding the specific intramuscular changes, especially from human tissue samples. The use of rodent models of collagen-induced arthritis to elucidate the intramuscular profile of major cellular role players and cytokine signalling in arthritis, may therefore contribute significantly to our understanding of the extent of dysregulation of intramuscular inflammation and muscle maintenance.

In our opinion, the collagen-induced arthritis (CIA) model is more physiologically relevant for this purpose than the complete Freund's adjuvant model, where inflammation is triggered in part by a bacterial insult, since bacterial infection is not accepted as a major general trigger for RA (although bacterial infection is implicated as role player in RA associated with periodontal disease specifically (Perricone *et al.*, 2019). While the use of Freund's adjuvant injected into the knee or ankle of mice successfully induced skeletal muscle pathology similar to that of RA patients (Steinz *et al.*, 2019), the type II collagen in complete Freund's adjuvant is the component responsible for triggering T-cells, B-cells and production of autoantibodies, simulating auto-immune disease mechanisms very similar to those observed in human RA development (Kannan, Ortmann and Kimpel, 2005; Asquith *et al.*, 2009). Although the model has its limitations, e.g. in terms of the time frame for disease and cachexia development, which is much faster than that of human RA, we have previously reported that the rodent CIA-model realistically mimicked the profile of rheumatoid cachexia in rats (Oyenihi *et al.*, 2019), justifying its use in the current context.

Although studies have focussed on different cell types/responses in isolation through cell culture models, literature still lacks a more comprehensively constructed picture in RA skeletal muscle. To our knowledge, there is no data to provide information on potential changes in relative distribution of different cellular role players in the skeletal muscle of RA patients, specifically those contributing to or affected by rheumatoid cachexia. Myoblasts, fibroblasts and macrophages interact with one another through the proteins (such as cytokines as growth factors) that they release (Ollewagen, Myburgh, *et al.*, 2021) to maintain healthy skeletal muscle. Dysregulation of this process could clearly be detrimental in a RA or chronic inflammatory environment. Therefore, this study aimed to quantify the relative distribution of different relevant cell types in skeletal muscle from a rodent CIA

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model, in parallel with intramuscularly secreted molecular messengers and indicators of arthritis-related cachexia.

## 4.3 Materials and methods

### 4.3.1 Ethics statement and animal handling

Following approval from the Stellenbosch University Animal Research Ethics Committee (Protocol number: SU-ACUD17-00034), twenty (20) female Sprague-Dawley rats weighing 180 – 200 g were obtained from the Stellenbosch University small laboratory animal breeding facility. The choice of using female rats only, is based on proof that female rodents are more susceptible to developing RA than males (Song *et al.*, 2015). Rats were housed in groups of 5 rats per cage in a temperature- and humidity-controlled room (23 ± 1°C, 40–60% humidity) with a set 12 h light-dark cycle (lights on at 6am) and fed standard commercially available rat chow and tap water *ad libitum*. After acclimatization, rats were randomly divided into two groups of 10 rats each – non-arthritis control (NC) and collagen-induced arthritis (CIA). All experimental animals received humane care according to the principles outlined in the National Research Foundation Guide for Care and Use of Laboratory Animals.

### 4.3.2 Collagen-induced arthritis model

The well-established rat collagen-induced arthritis (CIA) method was used to induce arthritis in the RA group, as previously described (Kannan, Ortmann and Kimpel, 2005; Asquith *et al.*, 2009; Song *et al.*, 2015; Oyenihi *et al.*, 2019). Briefly, bovine heterologous type II collagen (Chondrex Inc., WA, USA) was dissolved in 0.01N glacial acetic acid (2 mg/ml), followed by the preparation of an emulsion using an equal volume of incomplete Freund's adjuvant (Chondrex Inc., WA, USA). The emulsion was injected intra-dermally twice just above the tail region of each rat under isoflurane anaesthesia, 7 days apart. Non-arthritis control rats were subjected to identical anaesthesia, but were not subjected to any injections. The peak of the acute disease in terms of immune response typically occurs at 3 weeks (acute disease) and maximal joint damage occurs at 5 weeks (Rajaiah and Moudgil, 2009), therefore 5 weeks post-induction was selected as sample collection time point for the

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current study. Therefore, after a 5-week experimental period, all rats were killed by guillotine decapitation. The *gastrocnemius* muscle was removed, weighed, and frozen in liquid nitrogen-cooled isopentane and then stored at -80$^{o}$C until subsequent analysis. This muscle was previously shown to be most severely affected by CIA, with fibre type not a major determinant of cachexia outcome (Oyenihi *et al.*, 2019).

Successful induction of arthritis was confirmed by anti-collagen antibody titre testing, as well as clinical symptoms (e.g. paw edema) as previously described (Oyenihi *et al.*, 2019). All 10 animals treated with incomplete Freund's adjuvant developed arthritis symptoms. Neither the date of onset of observable symptoms such as edema, nor symptom severity (e.g. the number of limbs affected), correlated with antibody titre, and was highly variable between individuals. Therefore, we did not employ a selection protocol based on observed symptoms, in an attempt to account for the inter-individual variation seen in RA. The full details of the confirmation tests employed have been published earlier (Oyenihi *et al.*, 2019). The muscle samples used for generation of data presented here, were obtained from the cited study, which was previously conducted in our group, but all data presented here are novel (i.e. there is no duplication of previously published data).

### 4.3.3 Muscle histology

Frozen *gastrocnemius* tissues were sectioned into 10 µm cross-sections using a cryostat (Leica CM1860 UV, Leica Biosystems Nussloch GmbH, Germany) at -25°C. To ensure consistency between samples, a predetermined, standardised section was cut off the proximal end of each sample before sectioning, so that all sections were obtained at similar locality (depth and distance from proximal end) within the muscle. This allowed sections from all samples to come from the centre of the muscle.

*Cell populations and cross-sectional area:* Tissue sections were fixed in 4% paraformaldehyde (158127, Sigma-Aldrich, USA), washed and blocked for 90 minutes in 5% donkey serum (S217G, Celtic Diagnostics, South Africa) in 1% bovine serum albumin (BSA, 10735086001, Roche, Germany), with 0.2% Triton-X-100 (X100, Sigma-Aldrich, USA). Sections were incubated with primary antibodies for Pax7 (1:50, Pax7, Dev. Studies Hybridoma Bank, USA), F4/80 (1:200, sc377009, Lot#I1317, Santa Cruz, USA), and α-smooth muscle actin (1:250, α-SMA,

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Lot#125M4797V, A2547, Sigma-Aldrich, Germany) in 1% BSA overnight at 4°C. After washing, secondary antibodies were added for 2 hours (Donkey Anti-mouse 555, 1:500, ab150110, Abcam). Tissues were stained with Agglutinin (W11261, Thermo Fisher Scientific, USA) and Hoechst stain (ab33342, Abcam, UK).

Imaging was performed on a Confocal Microscope (Carl Zeiss LSM 780, Zeiss, Germany) and analysed on Zen 2011 software (Zeiss, Germany). The EC Plan-Neofluar 10x/0.3 M27 and Plan-Apochromat 20x/0.8 M27 objectives were used to acquire images at 100x and 200x magnification respectively. (Magnification represented as ocular lens (10x) multiplied by objective lens (10x/20x)).

*Image analysis:* For each animal, two cryosections were prepared, with one field of view analysed per section. (Both image acquisition and image analysis were performed on blinded samples.) Using ImageJ software (version 1.49, Wayne Rasband), the CSA of 50 fibres per sample was measured using the free-hand outline tool. The cell counter tool was used to count the number of positively stained macrophages, satellite cells, myofibroblasts, myonuclei, as well as the number of fibres per field of view. Cells were determined by positive stain of both the antibody and Hoechst. Images acquired at 100x magnification were used for CSA measurements; 200x magnification were used for all cell counts.

### 4.3.4 Cytokine and growth factor analysis

30 - 40 mg of muscle tissue was added to RIPA-based lysis buffer (containing 2X Complete Protease Inhibitor, 1X Complete Phosphatase Inhibitor, Roche) and homogenised using the PolyTron Manual Disperser (Kinematica, AG). After centrifugation, the supernatant was removed and used as the final protein sample. Protein concentrations were determined with a commercial Bicinchoninic acid (BCA) kit (BCA protein assay, Thermo Fischer Scientific, USA) according to the manufacturers' guidelines. 20 µg of protein from each sample was used for electrophoresis on polyacrylamide gels consisting of a 12% separating gel (0.39 M Tris-HCl (pH 8.8), 30% Acrylamide, 1 % SDS, 1% APS, 0.07% TEMED) and a 4% stacking gel (0.125 M Tris-HCl (pH 6.8), 12.5% Acrylamide, 1% SDS, 1% APS, 0.1% TEMED). Post electrophoresis gels were transferred onto a nitrocellulose membrane (GE Healthcare, Life science, RPN 3032D, UK) via a Turbo-blot transfer system (Bio-Rad, USA). After blocking for 1 hr with either 5% fat free milk in TBS-T or 1%

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BSA-TBS-T, membranes were incubated with primary antibodies in either 5% fat free milk-TBS-T or 1% BSA (Bovine Serum Albumin Fraction V,Roche, USA) in 1xTBS-T at 4$^{o}$C overnight. Primary antibodies included TNF-α (1:1000, NB600-587, Lot#41630, Novus Biologicals), IL-1β (1:1000, NB600-633, Lot#33815, Novus Biologicals), IL-6 (1:1000, NB600-1131, Lot#38801, Novus Biologicals), NFκB (1:1500, ab16502, Lot#GR3266473-1, Abcam), IL-10 (1:1000, ab9969, Lot#GR40933-42, Abcam), Id2 (1:1000, ab166708, Lot#GR3364842-1, Abcam), Myogenin (1:1000, sc12732, Lot#G0510, Santa Cruz), MyoD (1:1500, m3512, Lot#10045966, DAKO), PCNA (1:1000, ab15497, Lot#GR305622-1, Abcam), Mafbx (1:1500, ab168372, Lot#GR3241581-6, Abcam), TGF-β (1:800, ab92486, Lot#GR312172-2, Abcam), bone morphogenetic protein-7 (BMP-7, 1:1000, NBP1-69126, Lot#QC1792-42005, Novus Biologicals), MIF (1:1000, ab65869, Lot#GR221952-35, Abcam), MCP-1 (1:1500, NBP1-07035, Lot#B-3, Novus Biologicals) and GAPDH (1:4000, ab9485, Lot#GR3243682-1, Abcam). Membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibody (Cell Signaling, 7074S, Lot#26 or 7076S, Lot#31) 1:15 000 in 5% fat free milk in TBS-T for 1 hour and reaction with enhanced chemiluminescence (ECL, SuperSignal West Femto Maximum Sensitivity Substrate, 34094, Thermo Scientific) enabled subsequent imaging using the Chemidoc MP and Image Lab software. Immunoreactive proteins were then quantified using the same software and were normalised against GAPDH staining and the reference sample. Selection of antibodies was performed after confirmation of specificity, based on specifications provided by manufacturers. Secondary antibodies were also validated by absence of non-specific binding. All proteins were detected at the predicted molecular weight.

### 4.3.5 Statistical analysis

Statistical analysis was performed on GraphPad Prism v.8. Shapiro-Wilk test for normality was used to determine whether parametric or non-parametric tests were required. T-tests were performed for comparison between groups (unpaired t-test for normally distributed data; Mann-Whitney test for non-parametric data). Correlation analysis was performed (Pearson correlation coefficient or Spearman rank for parametric and non-parametric data respectively). $p<0.05$ was considered statistically significant.

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## 4.4 Results

*Gastrocnemius* muscle of rodents subjected to CIA exhibited a 57% reduction in CSA ($p<0.0001$) when compared to non-arthritic controls (Figure 4.1).

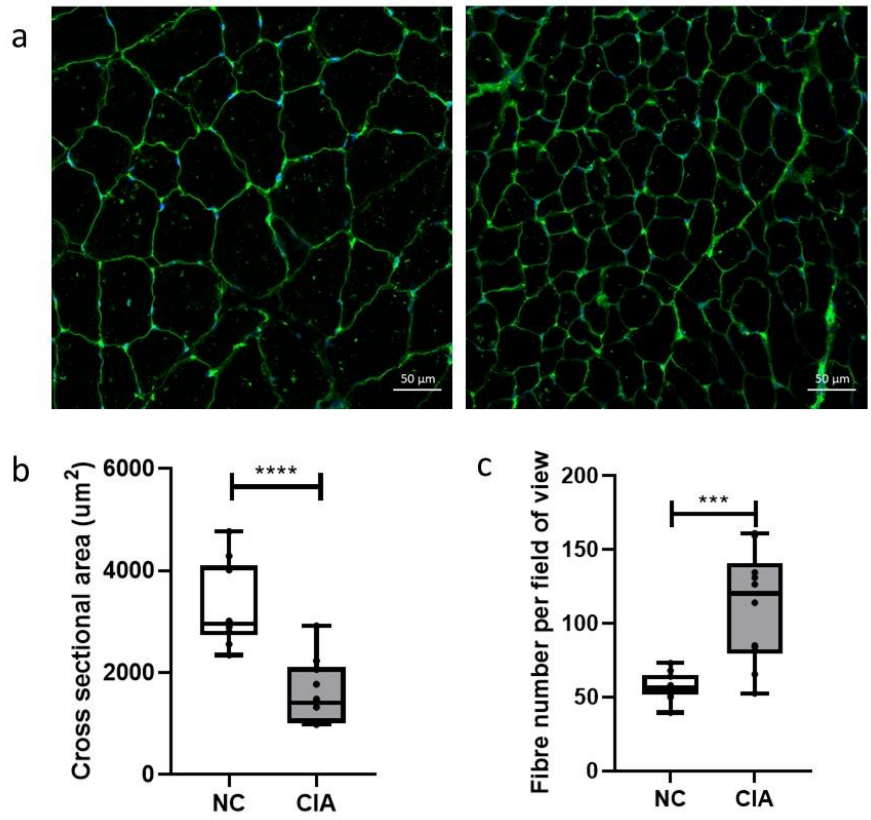


*Figure 4.1: Representative immunofluorescent images (a) and quantitated data (b) comparing cross-sectional areas of non-arthritic control (NC) versus collagen-induced arthritis (CIA) gastrocnemius muscle. n=10 per group. 100x magnification. Scale bar represents 50µm. Statistical analysis: Unpaired t-test. p<0.0001. Data represented as box and whisker plots indicating the highest and lowest values, the median and the interquartile range, as well as individual data points.*

Absolute counts of intramuscular abundance of cells and nuclei (Figure 4.2) revealed significant increases in the number of satellite cells, myofibroblasts, macrophages ($p<0.01$) and myonuclei ($p<0.001$) per field of view in the CIA *gastrocnemius* when compared to non-arthritic controls. When expressing cell and myonuclei counts

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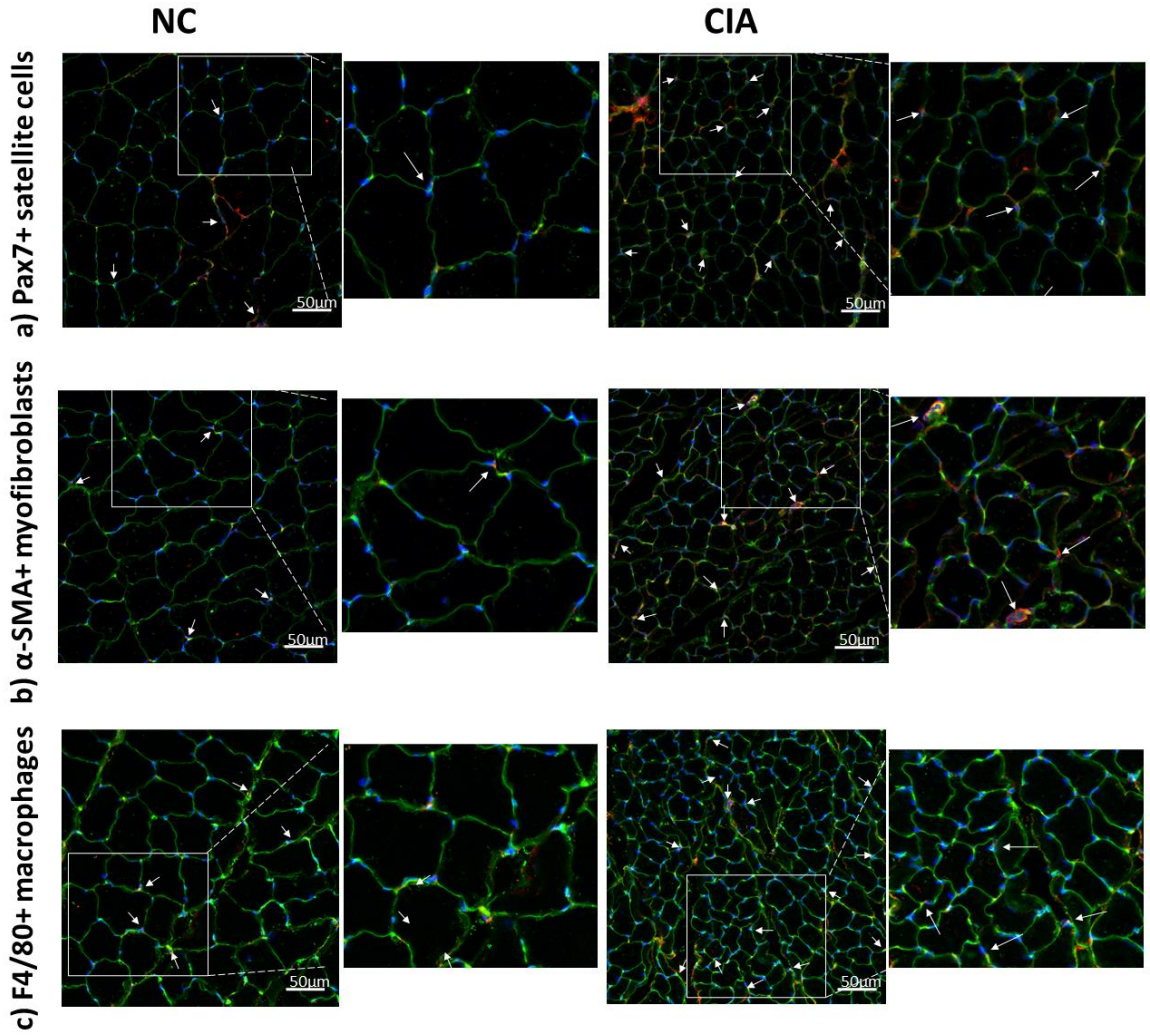
relative to number of myofibres per field of view, the CIA-associated increases were no longer evident.




d)

| Type | Number per field of view (NC) | Number per field of view (CIA) | Number per fiber (NC) | Number per fiber (CIA) |
|---|---|---|---|---|
| **Satellite cells** | 13.90 ± 1.48 | 23.30 ± 4.04 ** | 0.39 ± 0.10 | 0.31 ± 0.12 |
| **Myofibroblasts** | 7.30 ± 0.27 | 14.60 ± 3.09 ** | 0.21 ± 0.05 | 0.21 ± 0.08 |
| **Macrophages** | 21.00 ± 6.77 | 44.10 ± 12.57 ** | 0.54 ± 0.09 | 0.55 ± 0.16 |
| **Myonuclei** | 108.80 ± 7.94 | 282.00 ± 68.39 *** | 2.04 ± 0.33 | 2.34 ± 0.24 |

*Figure 4.2: Representative images (a-c) and quantification of (d) satellite cells, myofibroblasts (including myonuclei number) and macrophages in control and CIA*

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*rodent gastrocnemius muscle, expressed both as average number of cells per field of view and average number of cells per myofiber. n=5 per group. Statistical analysis: Unpaired t-test (normally distributed) or Mann-Whitney test (not normally distributed). **, p<0.01; ***, p<0.001 when compared to non-arthritic controls. Data expressed as mean ± SD. Magnification 200x. NC = non-arthritic control, CIA = collagen-induced arthritis.*

Western blot analysis of intramuscular cytokine levels (Figure 4.3) indicated an increase in pro-inflammatory and a decrease in anti-inflammatory cytokines in the CIA rodent muscle compared to non-arthritic controls. Specifically, there was a significant increase in TNF-α ($p<0.01$), whereas there was a significant reduction in IL-10 ($p<0.05$). The other pro-inflammatory cytokines did not exhibit significant changes, this included this included IL-1β ($p=0.21$), IL-6 ($p=0.17$), and NFκB ($p=0.14$). A noteworthy observation was that CIA did not seem to have an effect on levels of MCP-1 ($p=0.71$) or MIF ($p=0.72$), cytokines specifically associated with macrophage migration into tissue.

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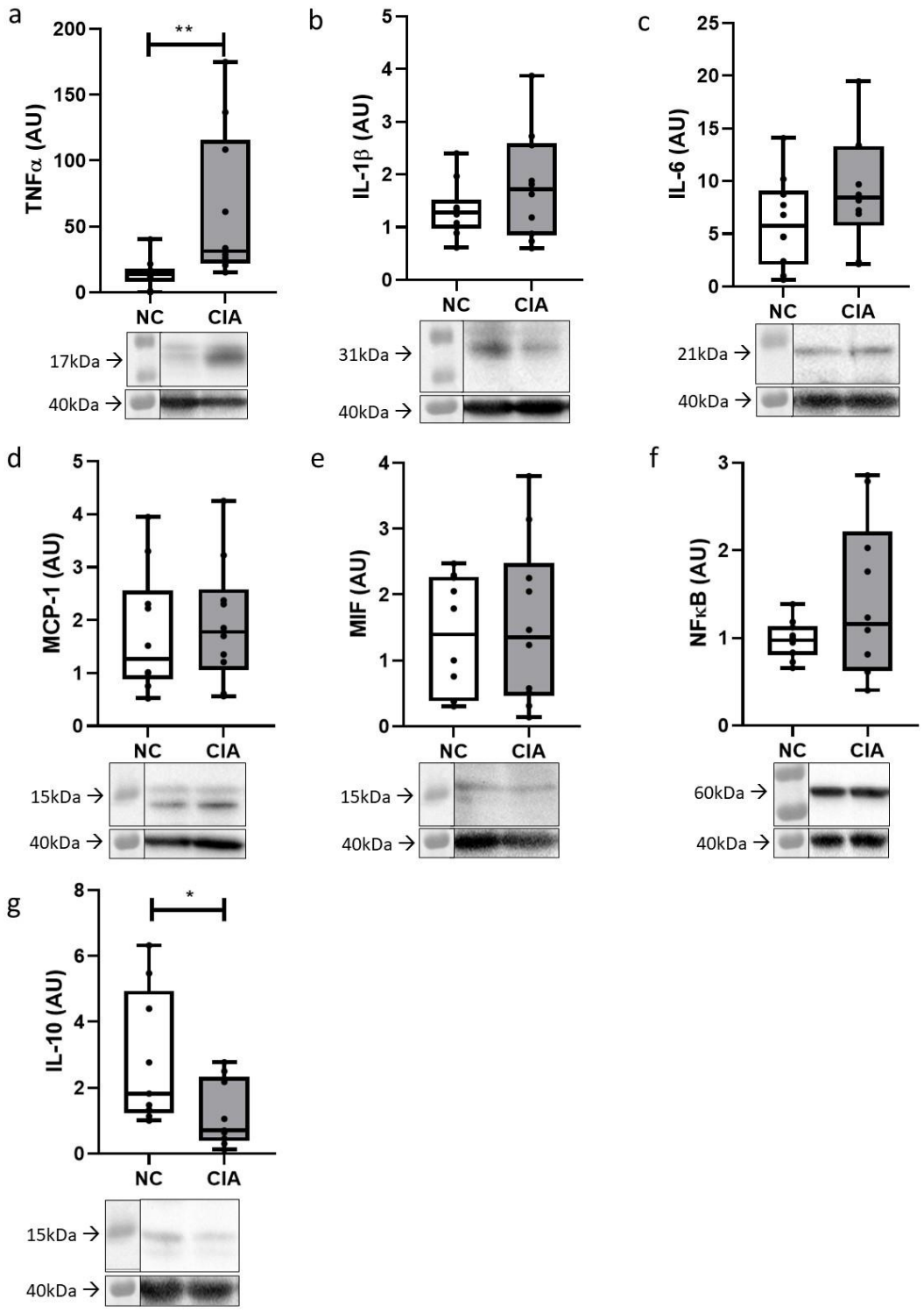


*Figure 4.3: Intramuscular pro- and anti-inflammatory cytokine levels in gastrocnemius muscle from normal control vs. collagen-induced arthritis rodents. a) TNFα; b) IL-1β; c) IL-6; d) MCP-1; e) MIF; f) NFκB; g) IL-10. n=10 per group. Statistical analysis: Unpaired t-test (parametric for b, c, d, e, f) or Mann-Whitney test (non-parametric for a, g). *, p<0.05; **, p<0.01. Data represented as box and whisker*

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*plots indicating the highest and lowest values, the median and the interquartile range, as well as individual data points. TNF-α = tumor necrosis factor-α; IL-1β = interleukin-1β; MCP-1 = monocyte chemoattractant protein; MIF = macrophage migration inhibitory factor; NF-κB = Nuclear Factor kappa-light-chain-enhancer of activated B cells. Representative images are provided for the protein of interest (top) and housekeeping protein (GAPDH, bottom) under each graph.*

The observation of relatively large interindividual variability in cytokine levels as well as in the CSA of the muscle fibres prompted further investigation of the correlation between cytokine levels and extent of muscle cachexia. Both TNF-α and NFκB ($p<0.01$ and $p<0.05$) demonstrated significant negative correlation to CSA in CIA rodents, whereby a smaller fibre CSA was correlated to a higher cytokine level. The opposite was demonstrated for IL-6 ($p<0.05$) where greater fibre CSA was correlated to higher cytokine levels (Figure 4.4c). Additionally, MIF was negatively correlated with CSA ($p=0.05$) in NC rodents, but not in the CIA rodents (Figure 4.4e). There was no significant correlation to CSA in the remaining NC samples.

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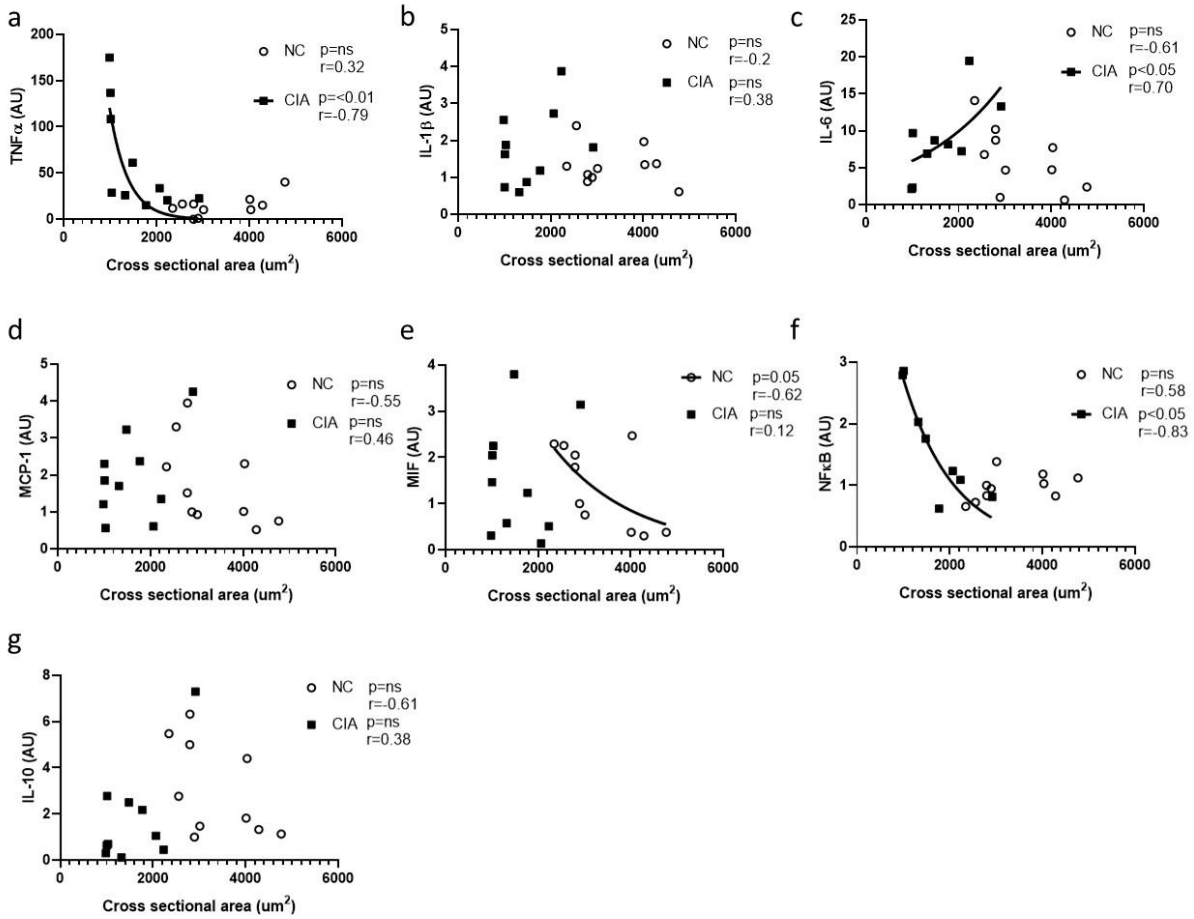


*Figure 4.4: Correlation between intramuscular cytokine levels and cross-sectional area of the gastrocnemius muscle in non-arthritic control (NC) and collagen-induced arthritic (CIA) rats - a) TNFα; b) IL-1β; c) IL-6; d) MCP-1; e) MIF; f) NFκB; g) IL-10. n=10 per group. Statistical analysis: Pearson linear correlation (parametric for b, c, d, e, f) or Spearman correlation (non-parametric for a, g). TNF-α = tumor necrosis factor-α; IL-1β = interleukin-1β; MCP-1 = monocyte chemoattractant protein; MIF = macrophage migration inhibitory factor; NF-κB = Nuclear Factor kappa-light-chain-enhancer of activated B cells.*

CIA was associated with a significantly higher expression of the markers indicative of cell proliferation, PCNA and MyoD when compared to controls (both P<0.05; Figure 4.5a and b). In addition, differentiation marker myogenin was significantly higher (p<0.05; Figure 4.5c) in CIA tissue as well as the growth factor TGFβ (p<0.05; Figure 4.5f). In contrast, CIA muscle exhibited higher levels of Id2, an inhibitor of differentiation, when compared to controls (p<0.01; Figure 4.5d). Mafbx, a marker of

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muscle wasting, seemed unaffected in CIA (Figure 4.5e). BMP-7, a stimulator of muscle growth, remained unchanged (Figure 4.5g).

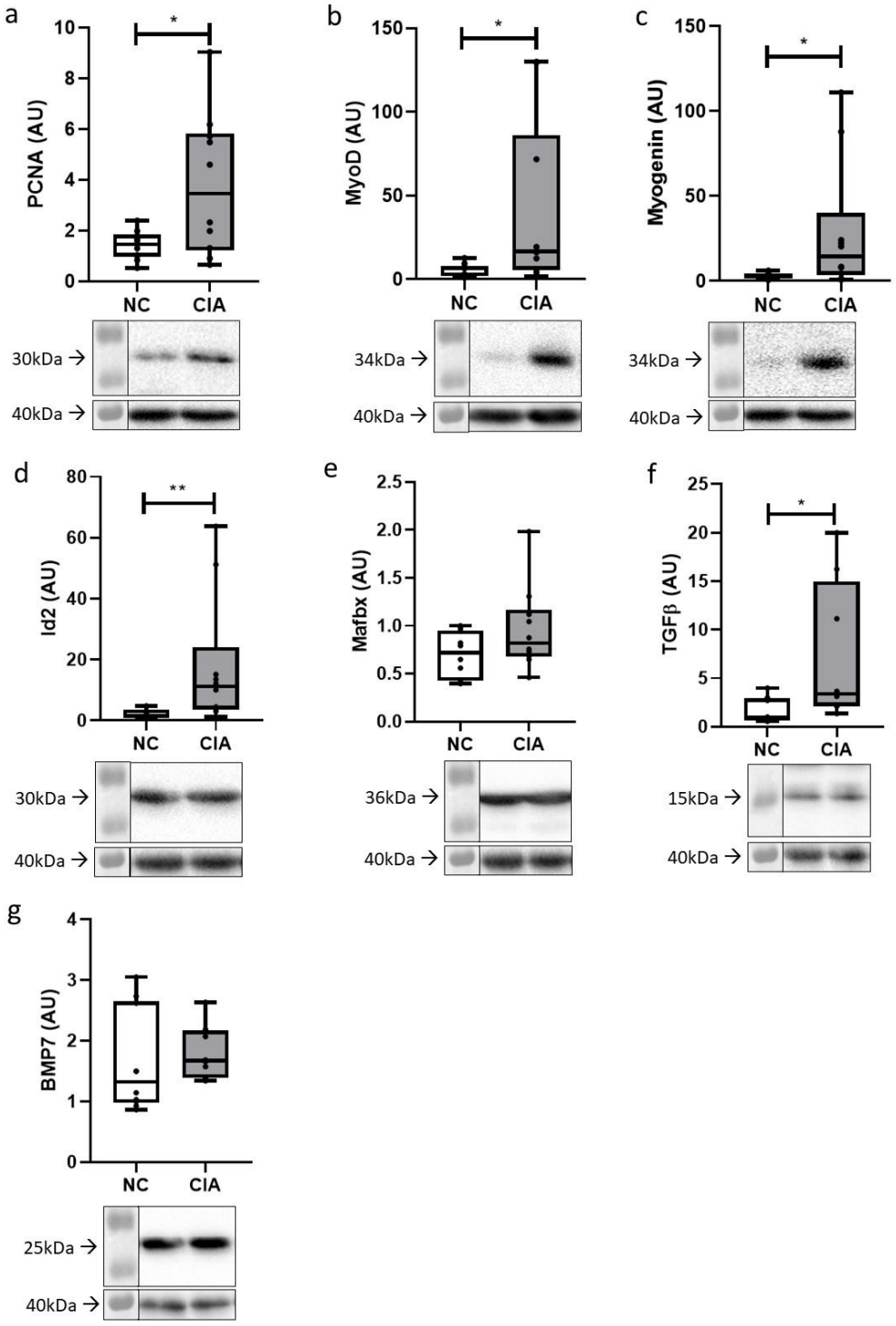


*Figure 4.5: Muscle proliferation and differentiation markers in control vs CIA gastrocnemius muscle. a) PCNA; b) MyoD; c) Myogenin; d) Id2; e) Mafbx; f) TGF-β;*

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*g) BMP-7. n=10 per group. Statistical analysis: Unpaired t-test (parametric for a, e) or Mann-Whitney test (non-parametric for b, c, d, f, g). *, p<0.05; **, p<0.01. Data represented as box and whisker plots indicating the range, the median and the 25-75 interquartile range, as well as individual data points. PCNA = proliferating cell nuclear antigen; TGF-β = transforming growth factor-β; Id2 = inhibitor of differentiation; Mafbx = muscle atrophy f-box; BMP-7 = bone morphogenetic protein. Representative images are provided for the protein of interest (top) and housekeeping protein (GAPDH, bottom) under each graph.*

Similar to correlations observed between cytokine levels and muscle fibre CSA, CSA also correlated with most muscle growth factors in the CIA rodents; with the exception of PCNA (which exhibited high variability) and BMP-7, all parameters assessed correlated negatively with CSA (Figure 4.6). This differed to the NC rodents, of which none were significantly correlated to CSA.

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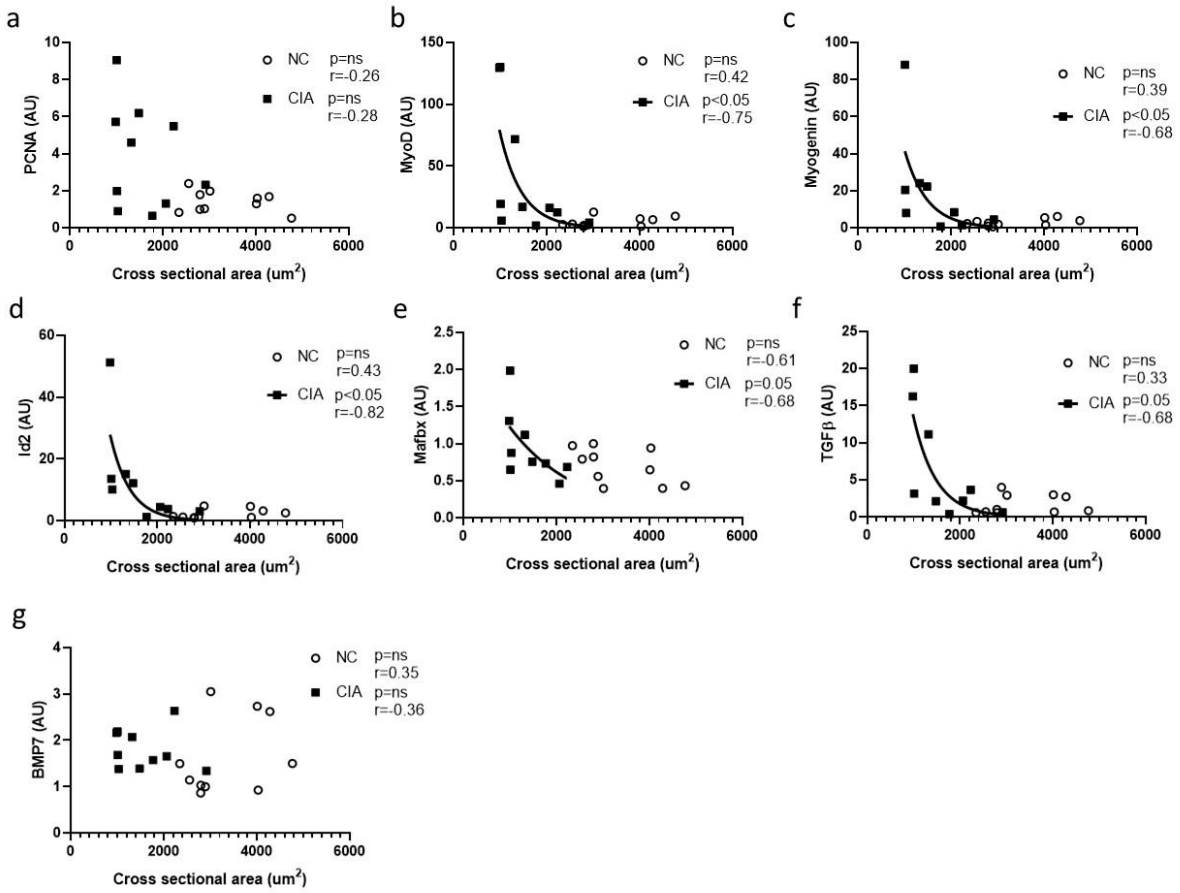


*Figure 4.6: Correlation between proliferation and differentiation markers and cross-sectional area of the gastrocnemius muscle. a) PCNA; b) MyoD; c) Myogenin; d) Id2; f) Mafbx; f) TGF-β; g) BMP-7. n=9-10 per group. Statistical analysis: Pearson correlation (parametric for a, e, g) or Spearman correlation (non-parametric for b, c, d, f). PCNA = proliferating cell nuclear antigen; TGF-β = transforming growth factor-β; Id2 = inhibitor of differentiation; Mafbx = muscle atrophy f-box; BMP-7 = bone morphogenetic protein.*

Taking into consideration the data from Figure 4.2 which indicates an increased fibre number per area of muscle, and the fact that RA-associated decreases in muscle fibre CSA did not appear to affect satellite cell number per fibre, it may be relevant to express parameters reported here, relative to number of nuclei available to receive these signals. As analysis by western blot does not account for fibre or cell number, we also expressed data for cytokines and growth factors relative to fibre number. When data were expressed relative to fibre number, many CIA-associated

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differences were no longer observed (Supp Fig 1). However, in the context of inflammation, TNF-α remained significantly elevated and IL-10 significantly decreased in CIA rodents (Figure 4.7a & b). Furthermore, BMP-7 demonstrated a new tendency for a decreased expression in CIA (Figure 4.7c), whereas muscle growth and differentiation markers, MyoD and myogenin, as well as the differentiation inhibitor Id2, remained significantly elevated (Figure 4.7d, e and f).

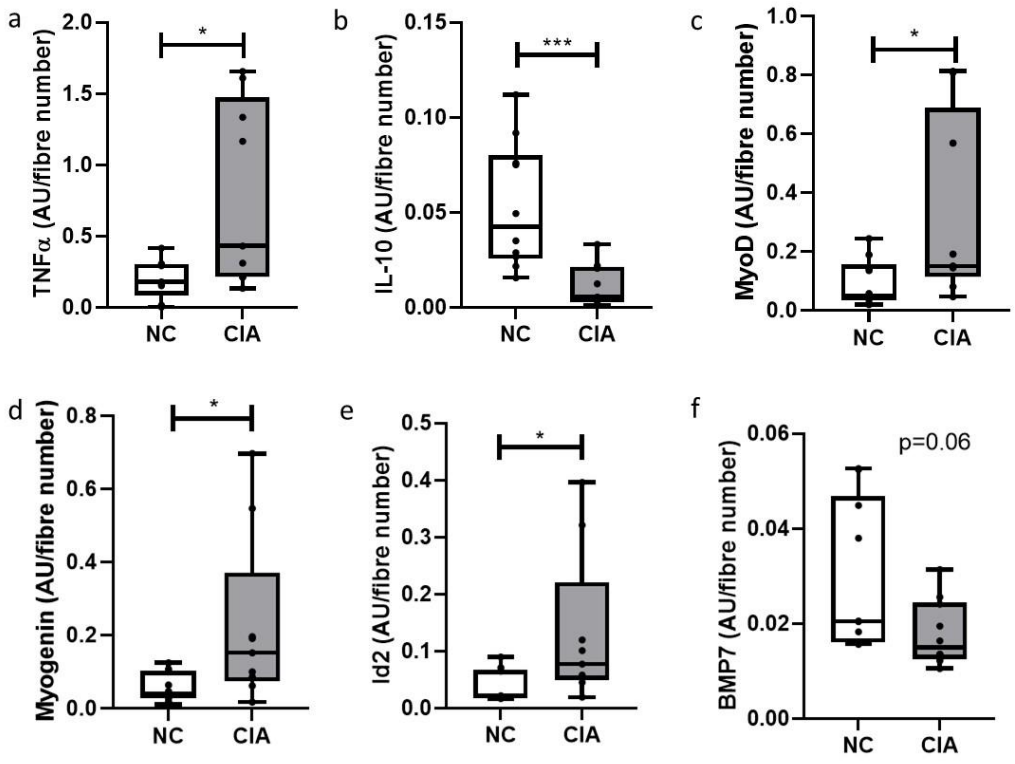


*Figure 4.7: Comparison of cytokines and growth factors taking fibre number into consideration. a) TNF-α; b) IL-10; c) BMP-7; d) Id2; e) MyoD; f) Myogenin. n=10 per group. Statistical analysis: Mann-Whitney test. *, p<0.05; **, p<0.01. Data represented as box and whisker plots indicating the highest and lowest values, the median and the interquartile range, as well as individual data points. TNF-α = tumor necrosis factor-α; IL-10 = interleukin-10; BMP-7 = bone morphogenetic protein; Id2 = inhibitor of differentiation.*

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## 4.5 Discussion

The current study assessed intramuscular cytokines in a rodent CIA model as a representative model of human rheumatoid cachexia. Research into the intramuscular cytokine concentrations in rheumatoid arthritis and CIA is extremely limited, with only one other paper, to our knowledge, measuring these levels in RA patients (Huffman *et al.*, 2017). Associated parameters assessed include downstream signalling and myogenic regulation. The novel research data presented here, highlights firstly the importance of assessing both TNF-α and IL-10 as indicators of the severity of this RA-induced co-morbidity. Secondly, current data suggest that expression of data relative to fibre number per area (as indirect indicator of signalling to nuclei of all other cell types involved, and as an indicator of signalling affecting the fibres themselves) may provide a more realistic picture of the multi-cellular signalling occurring in RA-affected skeletal muscle.

In terms of the severity of cachexia demonstrated in the CIA model, immunofluorescence analysis of muscle cross-sections demonstrated a significant reduction in CSA. This is in line with data reported by other groups in both the rodent CIA (Filippin *et al.*, 2013; Horai *et al.*, 2013) and human RA models (Helliwell and Jackson, 1994; Matschke, Murphy, Lemmey, P. Maddison, *et al.*, 2010), as well as with a previous report from our group, demonstrating an overall left shift in fibre size distribution in multiple muscle types (Oyenihi *et al.*, 2019). This, along with the loss in muscle mass itself, is one of the defining characteristics of cachexia, an occurrence in both early- and late-stage RA.

Assessment of cellular profile changes in RA muscles is also lacking, with early studies merely indicating presence of inflammatory infiltrate and fibrosis but not measuring the extent of cell type involvement (Finol *et al.*, 1988). Here, we demonstrate an overall increased presence of satellite cells, macrophages, myofibroblasts, and myonuclei per volume of CIA *gastrocnemius* muscle when compared to controls. However, this difference is not maintained when considering the numbers of these cells on a “per fibre” basis. The satellite cell data corresponds with a study of human RA-affected muscle biopsies, where satellite cell number per fibre were also not increased in response to the disease (Boutrup *et al.*, 2018). Current data suggest that counting of cells in the muscle tissue does not give the full

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picture and the signalling molecules should be taken in account to measure the processes predominating in the muscle.

It is widely known that pro-inflammatory cytokines, such as TNF-α and IL-1β, play a role in the pathophysiology of RA to the extent that a number of therapies have been designed to solely target these cytokines (Dayer, 2003; Fionula M. Brennan and McInnes, 2008). However, investigation into these cytokines within the muscle of rheumatoid cachexia models is limited, with most studies detecting increases in plasma or synovial fluid pro-inflammatory cytokines and reductions in anti-inflammatory cytokines (Roubenoff *et al.*, 1994). It has also been suggested that TNF-α plays a role in triggering inflammation locally and systemically, whereas IL-1β is more involved in local processes of cartilage and bone destruction (Dayer, 2002), another reason why IL-1β may remain low in muscle tissue compared to TNF-α. In response to an insult, TNF-α is released by circulating macrophages, which further stimulate the production and release of more TNF-α as well as IL-1. IL-1 then induces the production of IL-6 (Ott *et al.*, 2007). The significant increase in TNF-α in the current study indicates the presence of active disease and the central role for TNF-α. Further highlighting the importance of TNF-α in the development of rheumatoid cachexia, it remains elevated when correcting data for fibre number per area of muscle, confirming the availability of relatively more TNF-α signalling per fibre and thus per macrophage, satellite cell and fibroblast. In addition, IL-10 emerges as another robust indicator of inflammatory status in arthritis muscle, while arthritis-ascribed differences in other pro-inflammatory cytokines disappeared when expressing data on a per fibre basis. In human RA, assessment of intramuscular cytokines has demonstrated elevated IL-6 concentrations in RA patients, with other cytokines not showing significant changes (Huffman *et al.*, 2017). Of particular interest, the study by Huffman et al (2017) determined that muscle cytokine levels did not correspond to cytokine levels in circulation, highlighting the importance of measuring intramuscular cytokine concentrations when assessing rheumatoid cachexia. In the current study, CSA and IL-6 showed a significant positive correlation in CIA. IL-6, as a myokine has exhibited both pro- and anti-inflammatory effects within the muscle (Muñoz-Cánoves *et al.*, 2013), as well as exhibiting both hypertrophic effects through the stimulation of satellite cell proliferation (Serrano *et al.*, 2008), and the promotion of atrophy. However, atrophy as a result of IL-6 is

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largely dose- and time-dependent (Haddad *et al.*, 2005). IL-6 would seem to correlate with a hypertrophy outcome in the current study. Indeed, it has been suggested that IL-6 is the predominant cytokine present in the later maintenance stage of RA compared to TNF-α in the earlier stages (Kung *et al.*, 2020). However, a longitudinal assessment is probably required before firm interpretation can be made.

In terms of muscle growth factors, there was an overall increase in proliferation markers PCNA and the early myogenic marker, MyoD, as well as the differentiation marker, myogenin, in the CIA *gastrocnemius* muscle indicating an attempt to repair damaged muscle or promote regrowth following atrophy. This has also been demonstrated at different stages of atrophy in another rodent study (Castillero *et al.*, 2009). MyoD and myogenin remain elevated when taking into consideration the fibre number, highlighting that these two proteins have an exaggerated effect in attempting to repair the muscle despite satellite cell numbers remaining the same. However, in the CIA tissue, Id2, a marker for inhibition of differentiation is also significantly upregulated. Due to its ability to bind to and inhibit MyoD and other myogenic regulatory factors, Id2 may be contributing significantly to the poor muscle outcomes seen in cachexia (Jen, Weintraub and Benezra, 1992; Liu *et al.*, 2002). However, alongside its actions as a negative regulator of the myogenic regulatory factor family, Id2 has also been implicated in apoptosis-related atrophy (Alway *et al.*, 2003), a factor that requires more elucidation in rheumatoid cachexia. Once again, these markers, along with the atrogin, Mafbx, showed negative correlation to CSA. Overall, we suggest that the damage to the muscle due to inflammatory infiltration and fibrosis (Oyenihi *et al.*, 2019), and increased muscle breakdown, all contribute to necrosis and muscle loss, which is then followed by an attempt for muscle repair to occur with increased proliferation and differentiation of satellite cells. However, differentiation is also being strongly inhibited resulting in poor regenerative ability, leading to smaller fibres. As severity of cachexia is increased, more repair is attempted and failed, as indicated by the correlation to CSA. In addition to this, the increased inflammation itself may lead to an increase in the proliferation of myoblasts within the muscle (Bencze *et al.*, 2012), whereas the reduced anti-inflammatory cytokines contribute to the reduced differentiation and regeneration (Arnold *et al.*, 2007), ultimately leading to smaller fibres. Additionally, TNF-α, which is significantly increased in this study, has been shown to stimulate the ubiquitin-

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proteasome pathway (Li *et al.*, 2005; López-Menduiña *et al.*, 2010). Both TNF-α and Mafbx follow the same trend in terms of correlation to CSA which may suggest that they are some of the many components working together in the development of cachexia, it possible that in this study, TNF-α is elevated first, and the increase in Mafbx may reach significance at a later stage in disease development. It is also possible that Mafbx is upregulated at earlier stages in the development of cachexia as a study on rat CIA indicated peaked Mafbx at day 15 (Castillero *et al.*, 2009). More information may be obtained from a longitudinal study design, which may shed more light on dynamic changes over the course of disease progression. BMP-7 is vital for the maintenance of muscle mass after disruption of the neuromuscular junction, and contributes to hypertrophy (Winbanks *et al.*, 2013). In the *gastrocnemius* of the CIA rodent, BMP-7 does not change, nor is it correlated to CSA. However, when taking fibre number into consideration, there is a trend for a decrease in BMP-7, highlighting another factor that may be acting directly on the muscle fibres to influence rheumatoid cachexia.

In addition to its ability to inhibit muscle differentiation and contribute to apoptosis-related atrophy, Id2 also plays a role in the regulation of fibrosis. Id2 enhances proliferation of various cell types but also mediates TGF-β (Izumi *et al.*, 2006; Yin *et al.*, 2019). While TGF-β is vital in muscle growth via satellite cell activation (Delaney *et al.*, 2017), it also largely contributes to the production of collagen to develop fibrosis. Here, TGF-β is significantly increased compared to normal controls, and follows the same negative correlation as many of the other proteins when compared to CSA. We suggest that Id2 may be contributing to the proliferation of a variety of cells, including myoblasts and fibroblasts, and may be regulated in combination with TGF-β. However, TGF-β appears to predominate based the continued increase and on the fibrotic content seen in a previous study (Oyenihi *et al.*, 2019). Additionally, BMP-7, a TGF-β antagonist, exerts anti-fibrotic effects (Wu and Hatzopoulos, 2019), a mechanism which may be lost by its reduction in skeletal muscle. BMP-7 also reduced monocyte infiltration and increased M2 macrophage concentrations and anti-inflammatory cytokine levels in a rodent model of carotid artery ligation (Singla, Singla and Wang, 2016). Considering its muscle growth and anti-fibrotic effects, as well as these potentially positive effects on inflammation, it may be useful to

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investigate BMP-7 as a potential treatment option in the context of rheumatoid cachexia.

## 4.6 Conclusion

Novel data reported here includes the first comprehensive assessment of muscle regenerative capacity and intramuscular cytokine profile in CIA. In addition, current data illustrate for the first time, the cellular profile of CIA muscle.

Based on the increases in proliferation and differentiation markers, data suggest that CIA-associated cachexic muscle remains capable of regeneration, and that the unresolved inflammation is the primary role player impairing the maintenance of muscle fibre CSA. In terms of identification of therapeutic targets, current data does not support an approach focusing on modulation of muscle regenerative capacity *per se*, and rather indicate that the primary focus should remain on resolution of the chronic inflammation itself. In this respect, data further suggest that IL-10 should be assessed in addition to TNF-α, as indicators of inflammation severity. On a more practical note, data presented suggest that expressing data on a per fibre basis, may present a clearer picture of the signalling events at a cellular level.

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# Chapter 5

Chapter to be submitted to Inflammopharmacology (impact factor: 4.07)

Title: A primary human triple cell co-culture model simulation of cellular signalling in the skeletal muscle of rheumatoid arthritis patients

## 5.1 Abstract

The loss of muscle mass in rheumatoid arthritis (RA), termed rheumatoid cachexia, is predicted to result from the complex interactions between different cell types involved in the maintenance of skeletal muscle mass, namely myoblasts, fibroblasts and macrophages. The complexity within the muscle is further highlighted by the incidence of non-responsiveness to current RA treatment strategies. This study aimed to determine differences in the cellular responses in a novel human primary cell triple co-culture model exposed to serum collected from non-arthritic controls (NC), RA treatment naïve (RATN), and RA treatment non-responding (RATNR) patients. Bone morphogenetic protein-7 (BMP-7) was investigated as a treatment option. Plasma analysis indicated that samples were indeed representative of healthy and RA patients - notably, the RATNR patients additionally exhibited dysregulated IL-6/IL-10 correlations. Co-culture exposure to serum from RATNR patients demonstrated increased cellular growth ($p<0.001$), while both hepatocyte growth factor ($p<0.01$) and follistatin ($p<0.001$) were reduced when compared to NC. Furthermore, decreased concentration of markers of extracellular matrix formation, transforming growth factor-β (TGF-β; $p<0.05$) and fibronectin ($p<0.001$), but increased collagen IV ($p<0.01$) was observed following RATNR serum exposure. Under healthy conditions, BMP-7 exhibited potentially beneficial results in reducing fibrosis-generating TGF-β ($p<0.05$) and fibronectin ($p<0.05$). BMP-7 further exhibited protective potential in the RA groups through reversing the aberrant tendencies observed especially in the RATNR serum-exposed group. In conclusion, exposure of the triple co-culture to RATN and RATNR serum resulted in dysregulated myoblast proliferation and growth, and ECM impairment, which was reversed by BMP-7 treatment.

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## 5.2 Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease whereby, in addition to joint damage, patients demonstrate changes to body composition which includes a reduction in skeletal muscle, with or without the increase in fat mass (Matschke, Murphy, Lemmey, P. Maddison, *et al.*, 2010; Rafaela C.E. Santo *et al.*, 2018). This condition is termed rheumatoid cachexia. Rheumatoid cachexia has been reported as early as 1988, with assessments of muscle biopsies from patients demonstrating atrophy within the fibres (Finol *et al.*, 1988). However, control of disease activity in RA patients undergoing treat-to-target therapy to control inflammation does not seem to improve the function or composition of the muscle (Lemmey *et al.*, 2016), indicating a need for further understanding of the cellular mechanisms at work.

Regulation of skeletal muscle is complex, with the involvement of numerous cell types and growth factors (Ollewagen, Myburgh, *et al.*, 2021). The skeletal muscle contains stem cells (termed satellite cells) which become activated, proliferate, differentiate and fuse to form new myofibres (Ten Broek, Grefte and Von Den Hoff, 2010). Amongst the growth factors involved, hepatocyte growth factor (HGF) stands out as an activator of satellite cells (Lindström, 2009) and the modulation of the myostatin/follistatin axis regulates atrophy (Cappellari, Mantuano and De Luca, 2020). Muscle cells adhere to the extracellular matrix (ECM), a vital component for development, functioning and signalling within the muscle. Although muscle cells themselves also secrete numerous ECM components, the main contributor to ECM formation are the fibroblasts (Grzelkowska-Kowalczyk, 2016). This process is largely regulated by transforming growth factor-β (TGF-β), a protein secreted by fibroblasts, myoblasts and macrophages. This third cell type, macrophages, are resident in the connective tissue surrounding the myofibers (Gumucio *et al.*, 2013). Upon injury/insult, these and other circulatory macrophages will be recruited to the site in order to contribute to the degeneration and regeneration process (Chazaud *et al.*, 2009). Pro- and anti-inflammatory macrophages are vital in different stages of the regenerative process, and the shift in macrophage phenotype from M1 to the tissue remodelling M2 phenotype is vital in repair (Garg, Corona and Walters, 2015).

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However, during chronic inflammation, as demonstrated in RA, the balance between M1 and M2, and specifically the persisting presence of M2b macrophages disrupts the delicate balance between catabolism and anabolism, and the proliferation and differentiation of satellite cells, ultimately resulting in muscle wasting (Ollewagen, Myburgh, *et al.*, 2021).

The use of *in vitro* models to study different cellular facets of physiology has contributed significantly to our understanding of RA disease processes, but unfortunately lacks the complexity and thus direct relevance of *in vivo* studies. Typically, *in vitro* models employ a monolayer of only a single cell type for a narrowly focused investigation. However, interaction with various other cell types - as was demonstrated via culture “contamination” by other cells in primary mixed cell models (Cooper *et al.*, 2004; Miki *et al.*, 2012) – significantly influences cellular responses, such as myotube formation in muscle cell cultures. On the flipside, data from *in vivo* human studies are more variable due to relative difficulty to limit confounders; the resultant relative lack of statistical power often prohibits firm conclusions from data. Considering these factors, employing an *in vitro* model using multiple human cell types to more accurately simulate the cellular niche and cellular responses, may prove beneficial in further understanding interactions in both a healthy and diseased muscle environment.

A particular benefit of such a co-culture model is the ability to employ intervention treatments to further probe signalling responses or to identify potential therapeutic or preventative modalities. We have identified bone morphogenetic protein-7 (BMP-7) – which belongs to the TGF-β superfamily and which are structurally related to growth and differentiation factors (Daans, Lories and Luyten, 2008; Singla *et al.*, 2012) – as potential intervention agent. BMP-7 was initially studied due to its involvement in osteoblast differentiation and bone formation. However, several BMPs exhibit multiple biological activities in different cell types (Weziskirchen and Meurer, 2013). Overall, research into the role of BMP-7 in RA is limited: while a few studies reported on its role in reducing joint destruction (Steenvoorden *et al.*, 2006; Takahashi *et al.*, 2011), no studies are available in the context of targeting rheumatoid cachexia. Based on the fact that the TGF-β signalling network functions as a major component in developing skeletal muscle tissue, it is likely that the BMP axis too, may play a pivotal role in muscle mass regulation. Indeed, injection of BMP-7 vectors into

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mouse muscle were reported to result in increased myofiber area and diameter (Winbanks *et al.*, 2013). BMP-7 has also been implicated in the resolution of inflammation (Singla, Singla and Wang, 2016). Furthermore, in a mouse model of renal fibrosis, BMP-7 treatment reduced the severity of fibrosis and reversed renal pathology (Zeisberg *et al.*, 2003). Similarly, RA synovial fluid-stimulated fibroblast-like synoviocytes (FLS) treated with BMP-7 exhibited inhibited production of α-SMA, a marker expressed on synovial lining myofibroblasts (Steenvoorden *et al.*, 2006). Lastly, in a model of zymosan-induced arthritis (ZIA), direct injection with BMP-7 into the affected knee inhibited the loss of cartilage matrix and reduced swelling, as well as attenuating cellular infiltration, reducing IL-1β and increasing IL-10 levels (Takahashi *et al.*, 2011).

In the current study, we developed a novel triple co-culture model using primary muscle fibroblasts, myoblasts and blood-derived polarized M1 macrophages collected from healthy human donors. These standardised cultures were then exposed to serum from healthy or RA patients to better understand the interactions of various cell types and molecular role players in the muscle environment under conditions of diseased systemic signalling. Secondly, we report on the capacity of BMP-7 to alter the responses of relevant cytokines and growth factors in rheumatoid cachexia.

## 5.3 Methods

### 5.3.1 Ethics statement

Ethical clearance for this study was obtained from the Stellenbosch University Health Research Ethics Committee (HREC) for the isolation of myoblasts and fibroblasts from healthy volunteers (reference N12/08/051) and the collection of blood from healthy and RA patients (reference HREC2-2020-13147). Biosafety clearance for the handling of BSL2 samples was obtained from the Biosafety and Environmental Ethics Committee at Stellenbosch University (reference REC:BEE:2020-18524).

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### 5.3.2 Participant recruitment for primary cell isolation

For the isolation of myoblasts and fibroblasts, muscle biopsies were obtained from the *vastus lateralis* muscle of healthy, normally active young participants who were neither diabetic, nor using anti-inflammatory medication, and who did not have recent muscle injury. For the isolation of primary monocytes, blood was obtained in EDTA-coated tubes from healthy, young participants who did not have chronic/acute infections or injuries, smoked, or used anti-inflammatory medication.

### 5.3.3 Participant recruitment for rheumatoid arthritis study

Predicting the response of patients to treatment has shown low success rates with a number of patients not responding to treatment, developing resistance or treatment related adverse events (Day, 2002; Lindberg *et al.*, 2010). Blood was obtained in EDTA-coated and SST tubes from RA patients and healthy participants based on the following criteria: RA patients that were either (1) treatment naïve (RATN) or (2) had moderate to severe, treatment non-responding RA (RATNR) were recruited from Winelands Rheumatology Centre, Stellenbosch, South Africa. Patients did not have additional acute/chronic infections, co-morbidities, juvenile onset RA or obesity. Healthy participants (non-RA control, NC) were age-matched and excluded according to the same criteria as above, with the addition of the use of anti-inflammatory medication as exclusion criterion in the healthy group. Six participants/patients were recruited per group.

### 5.3.4 Patient/participant plasma analysis

RA patient and healthy participants plasma was collected in EDTA tubes and centrifuged at 400 x *g* for 10 minutes at room temperature. Plasma was analysed using the Milliplex human cytokine magnetic bead panel carried out according to the manufacturer's instructions. The following analytes were assessed: TNF-α, IL-1β, IL-1RA, IL-6 and IL-10 (HCYTOMAG-60K; Merck Millipore, Darmstadt, Germany).

### 5.3.5 Primary cell isolations

Primary fibroblasts were isolated according to the previously established consecutive pre-plating protocol (Goetsch *et al.*, 2015). Briefly, biopsy tissue was digested in collagenase/dispase solution (10269638001, Sigma Aldrich) and placed in ECL-pre-

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coated flasks. After allowing primary fibroblast attachment for 1 hour, media with unattached cells was removed was discarded. Primary myoblasts were isolated according to the micro-explant technique (Gudagudi *et al.*, 2020), in which pieces of muscle biopsy were plated on entactin-collagen IV-laminin (ECL; 08-110, Merck, USA) pre-coated plates and myoblasts were allowed to migrate out of the tissue. Cells from the third and fourth sub-culture were used, as initial cells were a combination of myoblasts and fibroblasts.

Prepared pure isolates of primary fibroblasts and myoblasts were cultured in complete Hams-F10 media (N6908, Sigma-Aldrich) supplemented with 20% foetal bovine serum (FBS; 10499-044, Life Technologies), 1% penicillin-streptomycin (P43333, Sigma-Aldrich) and 2.5 ng/ml human recombinant fibroblast growth factor (hrFGF; G5071, Promega). After sufficient stocks were created, primary myoblast and fibroblast media was converted to RPMI 1640 media (with Glutamax; 61870010, Gibco) for consistency between the three cell-types.

Primary monocytes were isolated from donated blood using a double gradient centrifugation protocol (Menck *et al.*, 2014). Monocytes were cultured in RPMI 1640 media containing 20% FBS and 1% penicillin-streptomycin in 24-well plates pre-coated with ECL. Cells were supplemented with 50 ng/ml of granulocyte macrophage colony-stimulating factor (GM-CSF; SRP3050, Sigma-Aldrich) to allow pre-differentiation to occur. Cells were allowed to adhere for 24 hours before the first media change, thereafter media was changed every 2 days (for 4 additional days). Cells were polarized to a M1 phenotype with 50 ng/ml GM-CSF, 50 ng/ml lipopolysaccharide (LPS; L2762, Sigma-Aldrich,) and 20 ng/ml interferon-γ (IFN-γ; I3265, Sigma-Aldrich) for 24 hours.

### 5.3.6 Cell phenotype confirmation

To confirm primary human myoblast (PHM) and primary human fibroblast (PHF) phenotype and culture purity, cells were fixed with 4% PFA, blocked, and stained overnight with desmin (ab15200, Abcam, UK) and fibronectin (sc80982, Santa Cruz, USA) at $4^0$C. Cells were then stained with fluorescence-labelled secondary antibodies (594 - 150064 and 488 - 150109, Abcam, UK) and Hoechst (ab33342 Abcam, UK), mounted with fluorescent mounting media (53023, DAKO, Denmark) and imaged on the Zeiss confocal microscope (Carl Zeiss LSM 780, Zeiss,

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Germany) at 200x magnification. PHM phenotype was confirmed by positive staining with desmin only, while PHF phenotype was confirmed by positive staining with fibronectin only (Figure 5.1).

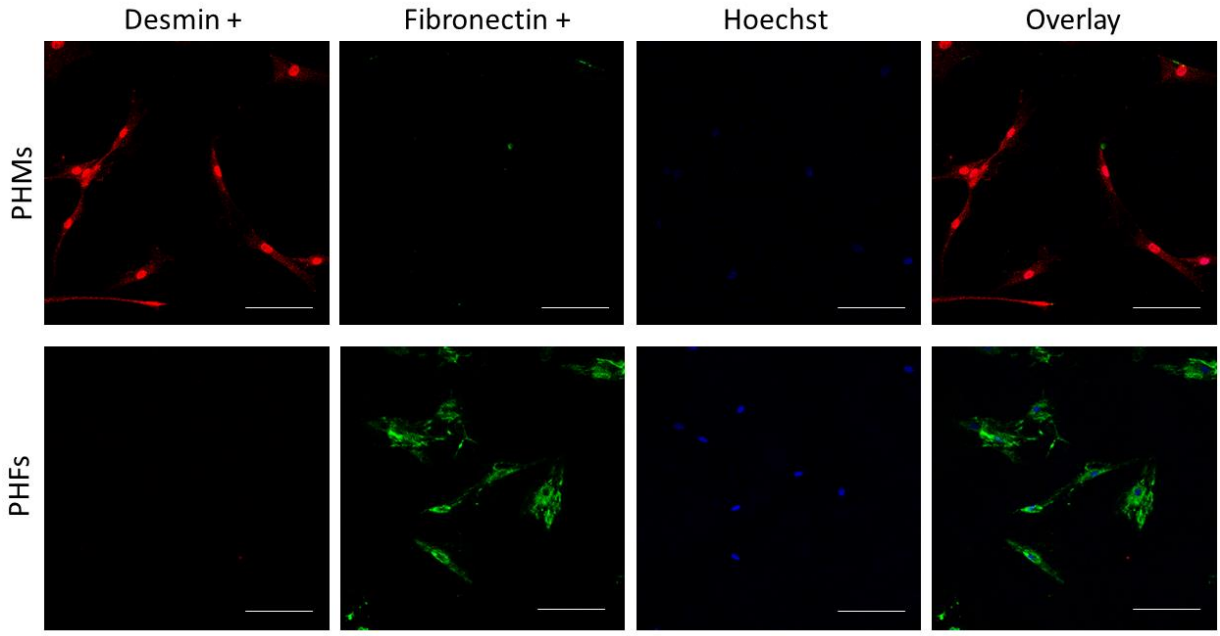


*Figure 5.1: Cell type confirmation of primary human myoblasts (PHMs) and fibroblasts (PHFs) staining with desmin (red) and fibronectin (green) respectively. Nuclei are visualised using Hoechst (blue). Images were captured at 200x magnification. Scale bar represents 100 µm.*

### 5.3.7 Triple co-culture with patient serum

Patient serum was collected in SST tubes and after allowing clotting for 30 minutes at room temperature, centrifuged at 1500 x *g* for 10 minutes. Media (RPMI 1640) was prepared with 20% patient serum and 1% penicillin-streptomycin. Primary myoblasts and fibroblasts were detached with trypsin (25200072, Gibco). Primary M1-polarised macrophages were detached with Accutase® (A6964, Sigma-Aldrich). Cells were plated on ECL pre-coated plates in the patient serum-containing media in the ratio of 40 000 macrophages:10 000 myoblasts:5 000 fibroblasts as determined with intramuscular cell staining in a rodent collagen-induced arthritis model (Ollewagen, Powrie, *et al.*, 2021; Chapter 4). (The number of macrophages were

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modified to twice the number present in RA rodents, to correct for lack of proliferation of the terminally differentiated macrophages over the course of the culture protocol.) Plates were shaken every 15 minutes over a 90 minute period to allow even distribution of cells. Triple co-cultures were prepared in duplicate for each patient and treatment condition.

### 5.3.8 BMP-7 treatment of various cell types

After 48 hours of exposure of triple co-cultures to patient serum-conditioned media, media was replaced with serum-conditioned media and cells treated with 750 ng/ml BMP-7 (prepared in $dH_2O$) for an additional 48 hours. The dose of 750 ng/ml was determined in a pilot dose response study using single cell cultures for all cell types (refer to Appendix E). After 48 hours, cell culture supernatants were removed and centrifuged at 500 x *g* for 5 minutes to remove remaining cells and debris. Images were taken on the Olympus microscope (CKX41, Olympus Corporation) on day 2 and day 4 at 40x and 100x magnification. In addition, 100x images were analysed using ImageJ to measure area fraction of cells (measure of confluence) within each field of view.

### 5.3.9 Supernatant analysis

Triple co-culture clarified supernatants were analysed using ELISA and Multiplex quantikine analyses as follows: follistatin (DFN00, R&D Systems), GDF-8/Myostatin (DGDF80, R&D Systems), Decorin (NBP3-08102, Novus Biologicals), Fibronectin (E-EL-H0179-96T, E-Lab Bioscience), collagen IV (E-EL-H0178-96T, E-Lab Bioscience), TGF-β magnetic Luminex (FCSTM17-01, R&D Systems), and magnetic Luminex for collagen I alpha 1, HGF, IL-1β, IL-6, IL-10, and TNF-α (LXSAHM-07, R&D Systems).

### 5.3.10 Statistical analysis

Statistical analysis was performed on GraphPad Prism v.8. Patient data and plasma results were assessed for normality using Shapiro-Wilk analysis. Data was analysed using a one-way ANOVA and Tukey's multiple comparisons for parametric data, and Kruskal-Wallis test with Dunn's multiple comparisons for non-parametric data. Correlations were performed using Pearson's correlation.

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## 5.4 Results

### 5.4.1 Patient group characterisation plasma cytokine profiles

As expected, patient groups exhibited variable and different duration of diagnosis periods, with treatment naïve patients reporting having RA for 2.33±3.67 years (4 out of 6 were recently diagnosed), and treatment non-responding patients having RA for 11.80±13.33 years. Affected joints included wrists, hands, elbows, ankles, and knees in both groups. Age and body composition did not differ significantly between groups: healthy controls (NC) were 50.2 ± 8.5 yr old (3 male, 3 female; BMI: 29.79 ± 4.57), treatment naïve (RATN) patients were 53.7 ± 18.9 yr old (1 male, 5 female; BMI: 30.79 ± 7.56), and RA treatment non-responding (RATNR) patients were 59.3 ± 14.2 yr old (1 male, 5 female; BMI: 24.89 ± 3.30). Of the recruited patients, 4 of the 6 treatment naïve patients stated they had a noticeable loss in muscle mass, whereas 2 of the 6 treatment non-responding patients confirmed noticeable muscle loss. However, no patients were assessed in a quantitative manner for muscle loss over the period of active disease.

Comparison of groups for patient plasma cytokines indicated limited statistically significant differences between groups, likely due to large variability amongst patients and a small sample size (Figure 5.2). Nevertheless, the general picture in both RA groups are in line with a relatively more pro-inflammatory state. In addition, RATNR patients had a significantly increased IL-6 plasma concentration ($p<0.05$) compared to RATN patients (Figure 5.2c).

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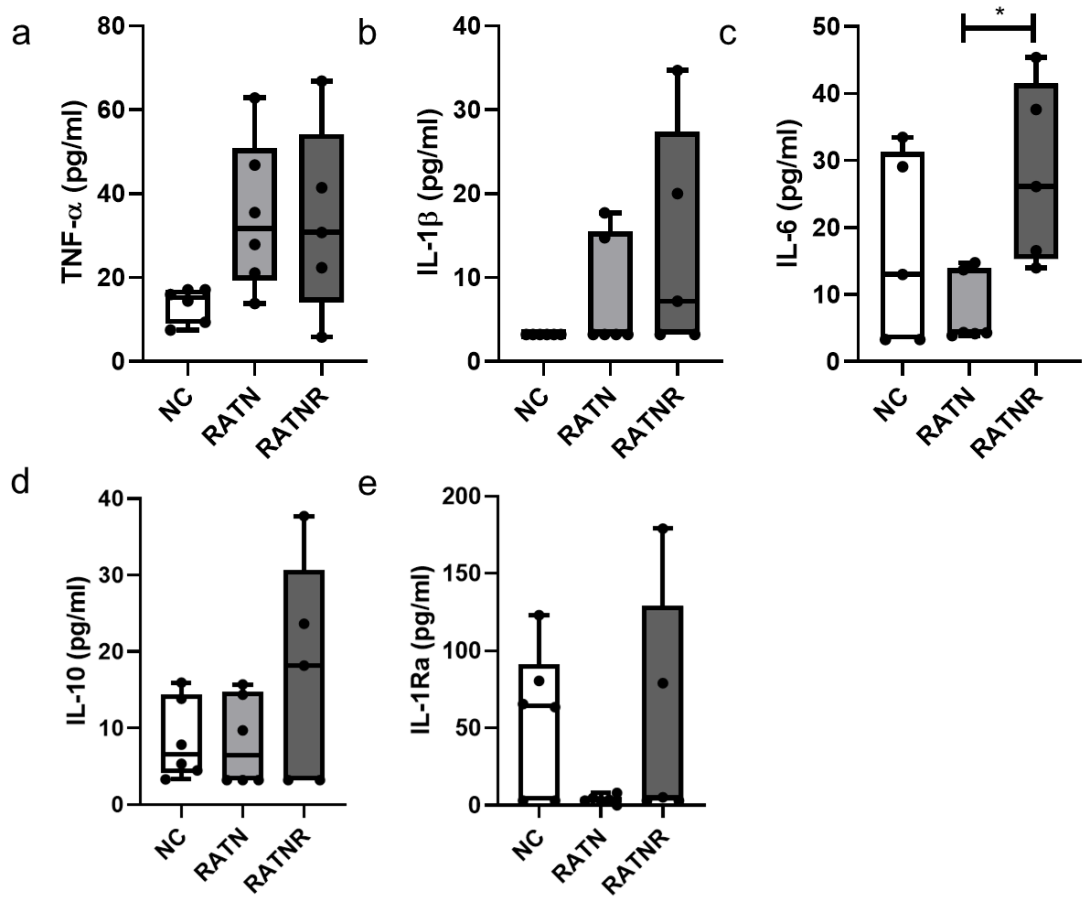


*Figure 5.26: Patient plasma cytokine concentration in healthy (NC), RA treatment naïve (RATN) and RA treatment non-responding (RATNR) patients. a) TNF-α; b) IL-1β; c) IL-6; d) IL-10; and e) IL-1Ra. Statistical analysis: one way ANOVA. * = p<0.05. n=6 per group. Data represented as box and whisker plots indicating the highest and lowest values, the median and the interquartile range, as well as individual data points. TNF-α = tumor necrosis factor-α; IL = interleukin.*

Correlation between IL-10 and IL-6 demonstrated a significant positive correlation in the NC group that was lost in the RATN group, whereas the RATNR group demonstrated a significant negative correlation (Figure 5.3).

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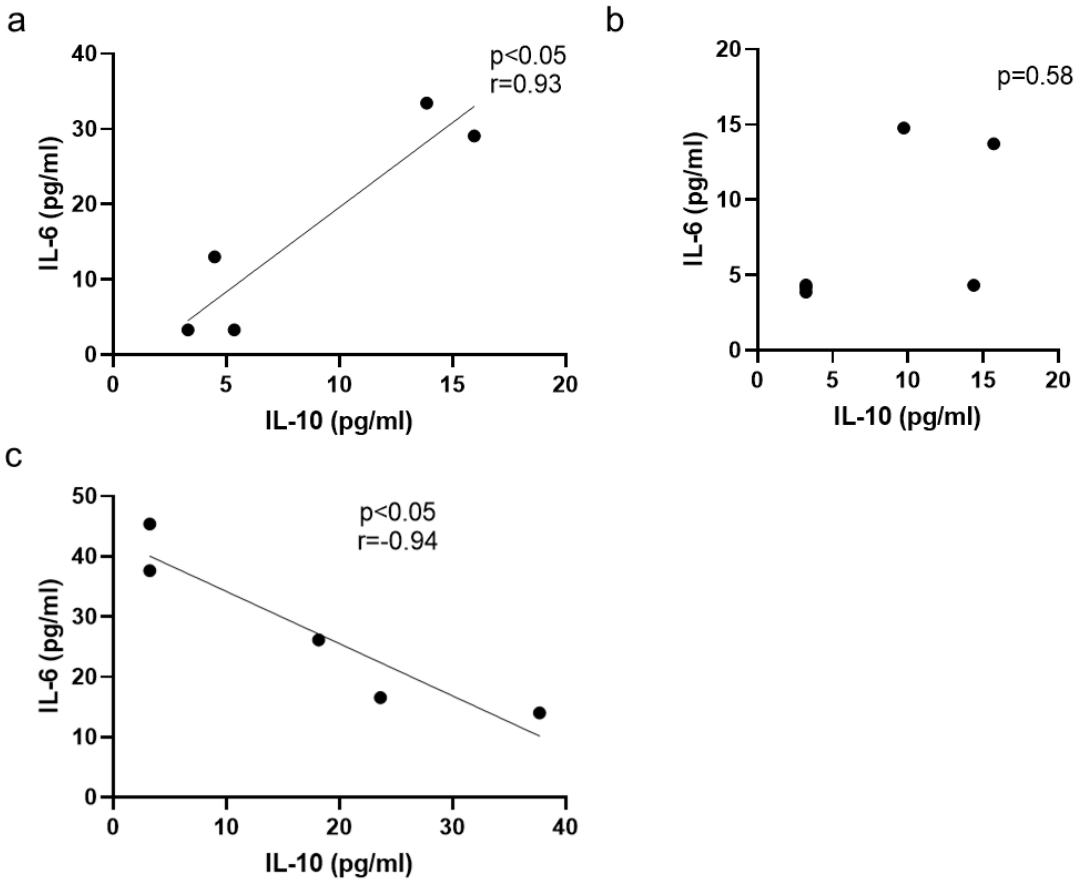


*Figure 5.3: Correlation between IL-10 and IL-6 in the different patient groups. a) non-arthritic control (NC); b) RA treatment naïve (RATN); c) RA treatment non-responding (RATNR). Statistical analysis: Pearson's correlation. n=5 per group.*

### 5.4.2 Triple co-culture responses

Qualitatively, healthy participant cells exhibited a unique response to the serums from different patient groups, as indicated by the representative images in Figure 5.4. Cells appeared to proliferate at different rates, as is evident from the differences in relative confluence - RATNR serum resulted in the fastest growth rate, resulting in cultures appearing fully confluent after 2 x 48 hours. Macrophages in the RATNR-exposed cultures appeared to demonstrate a greater extent of activation compared to both other groups. In the RATN group, patient serum seemed to result in clustered growth patterns of cells.

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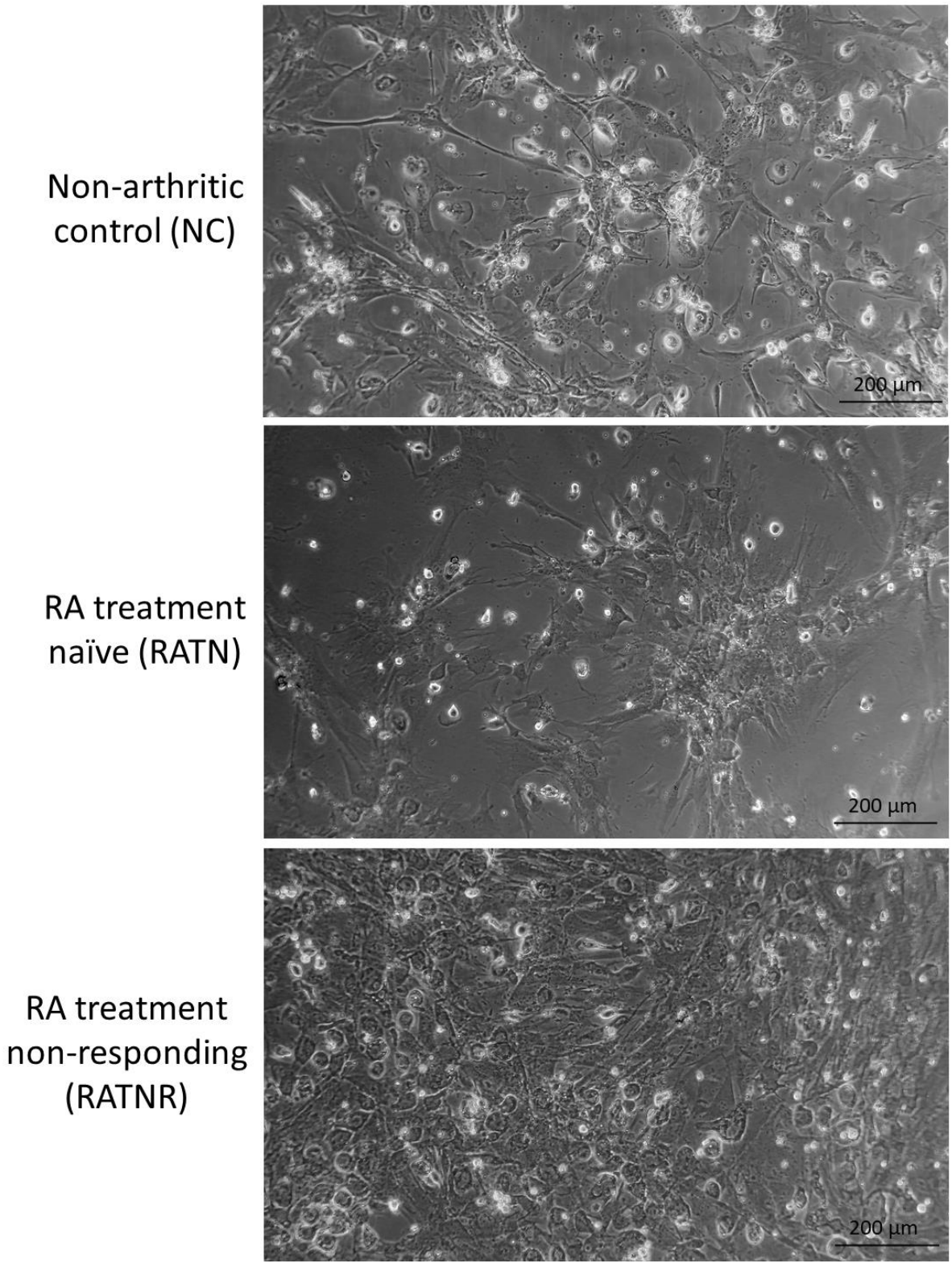


*Figure 5.4: Representative images of the triple cell culture model indicating examples of the different observed responses to the patient serum. Images taken at 100x magnification. Scale bar represents 200 µm.*

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Quantification of area fraction (%) as measure of confluence confirmed that cultures treated with RATNR patient serum proliferated more extensively and therefore exhibited a significantly higher area fraction than other patient groups, both with and without BMP-7 treatment (Figure 5.5).

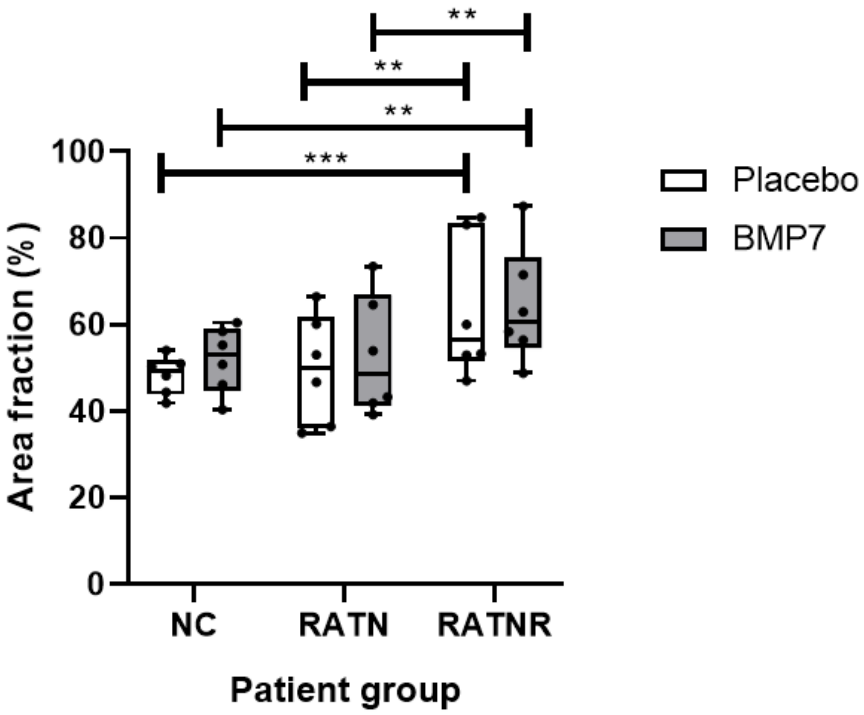


*Figure 5.5: Percentage area fraction taken up by the triple culture cells in the field of view, comparing the cultures exposed to serum from healthy control, treatment-naïve and treatment non-responding RA patients, with or without BMP-7 treatment (750 ng/ml). n=6. Statistical analysis: Two-way ANOVA with Tukey's multiple comparisons. ** = p<0.01; *** = p<0.001. Data represented as box and whisker plots indicating the highest and lowest values, the median and the interquartile range, as well as individual data points.*

Luminex analysis of culture supernatants generally demonstrated very low levels of IL-1β and IL-10. IL-10 concentration was below detectable limits in all groups, while IL-1β concentration only measurable in 1 RATN exposed culture and 2 RATNR exposed cultures (data not shown). This data was thus excluded from interpretation. Similarly low levels of TNF-α were detected across all groups. IL-6 seemed to be

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secreted in relatively high quantities, but appeared similar between groups (Figure 5.6).

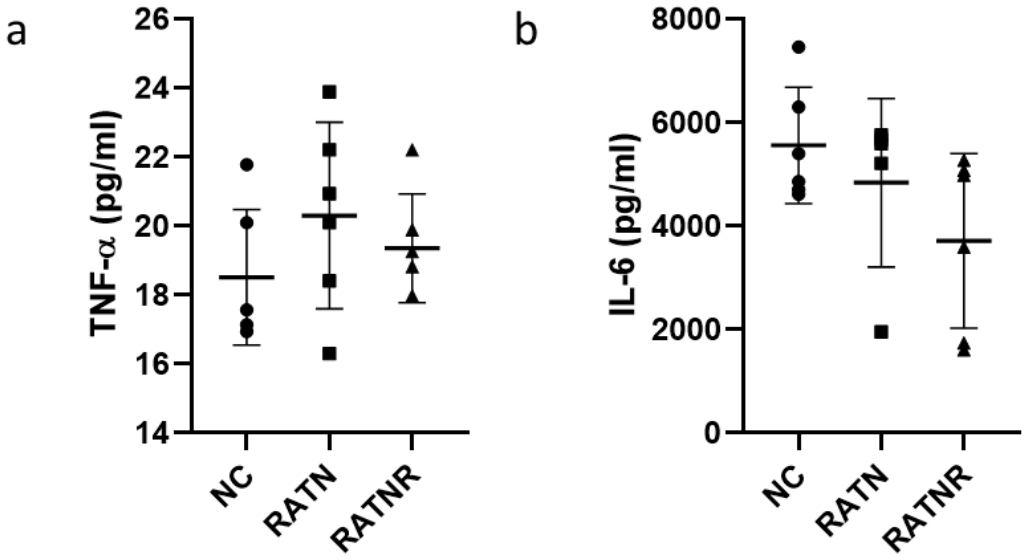


*Figure 5.6: Triple culture supernatant cytokine concentration comparing the conditioned media exposed to healthy (NC), RA treatment naïve (RATN), and RA treatment non-responding (RATNR) patients' serum. a) TNF-α; b) IL-6. Statistical analysis: one-way ANOVA. n=6 per group. Data represented as mean ± SD. TNF-α = tumor necrosis factor-α; IL = interleukin.*

More significant differences between groups were evident for muscle growth factors (Figure 5.7). HGF concentration was significantly reduced in the media exposed to RATNR serum when compared to the control, while follistatin concentration was significantly reduced in both the RATN ($p<0.01$) and RATNR serum exposed groups ($p<0.001$) when compared to the control. Myostatin demonstrated no significant differences between the groups.

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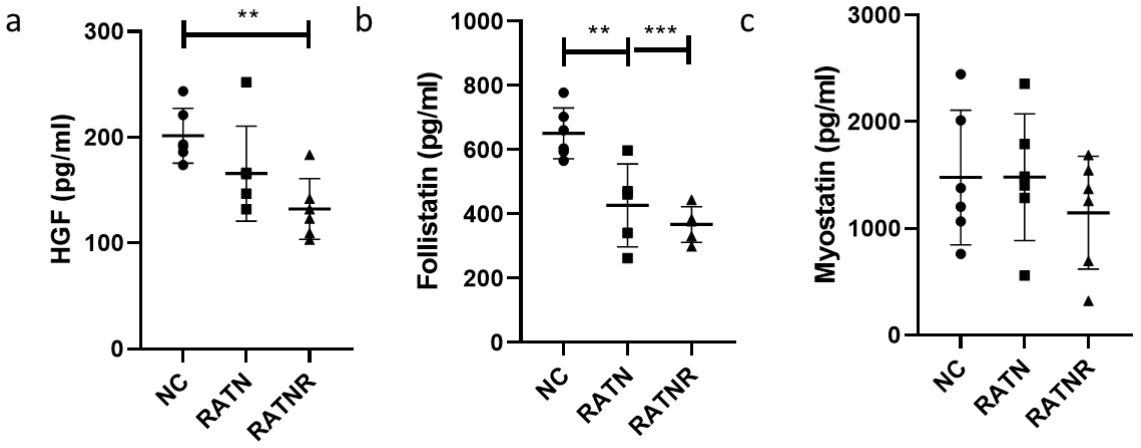


*Figure 5.7: Triple culture supernatant muscle growth factor concentration comparing healthy (NC), RA treatment naïve (RATN), and RA treatment non-responding (RATNR) patients. a) HGF; b) Follistatin; c) Myostatin. Statistical analysis: one-way ANOVA (parametric: c) or Kruskal-Wallis test (non-parametric: a, b). ** = p<0.01; *** = p<0.001. n=6 per group. Data represented as mean ± SD. HGF = hepatocyte growth factor.*

Turning attention to fibroblast growth factors, TGF-β concentration was significantly reduced in the RATNR serum exposed group when compared to both the control group and RATN group (Figure 8a). Decorin levels were similar in all groups (Figure 5.8b), while fibronectin concentration was significantly lower than NC in the RATN and RATNR groups, as well as lower (p=0.05) in RATNR when compared to RATN (Figure 5.8c). Collagen 1a1 levels were greater than the detectable limit for all NC samples, but fell within the detection range for the kit for at least half the samples of the RATN and RATNR groups, suggesting that these groups may exhibit lower collagen 1a1 than the healthy controls (Figure 5.8d). Collagen IV concentration appeared higher in response of both RA serum groups, with a statistically significantly increase in the RATNR group compared to healthy controls (Figure 5.8e).

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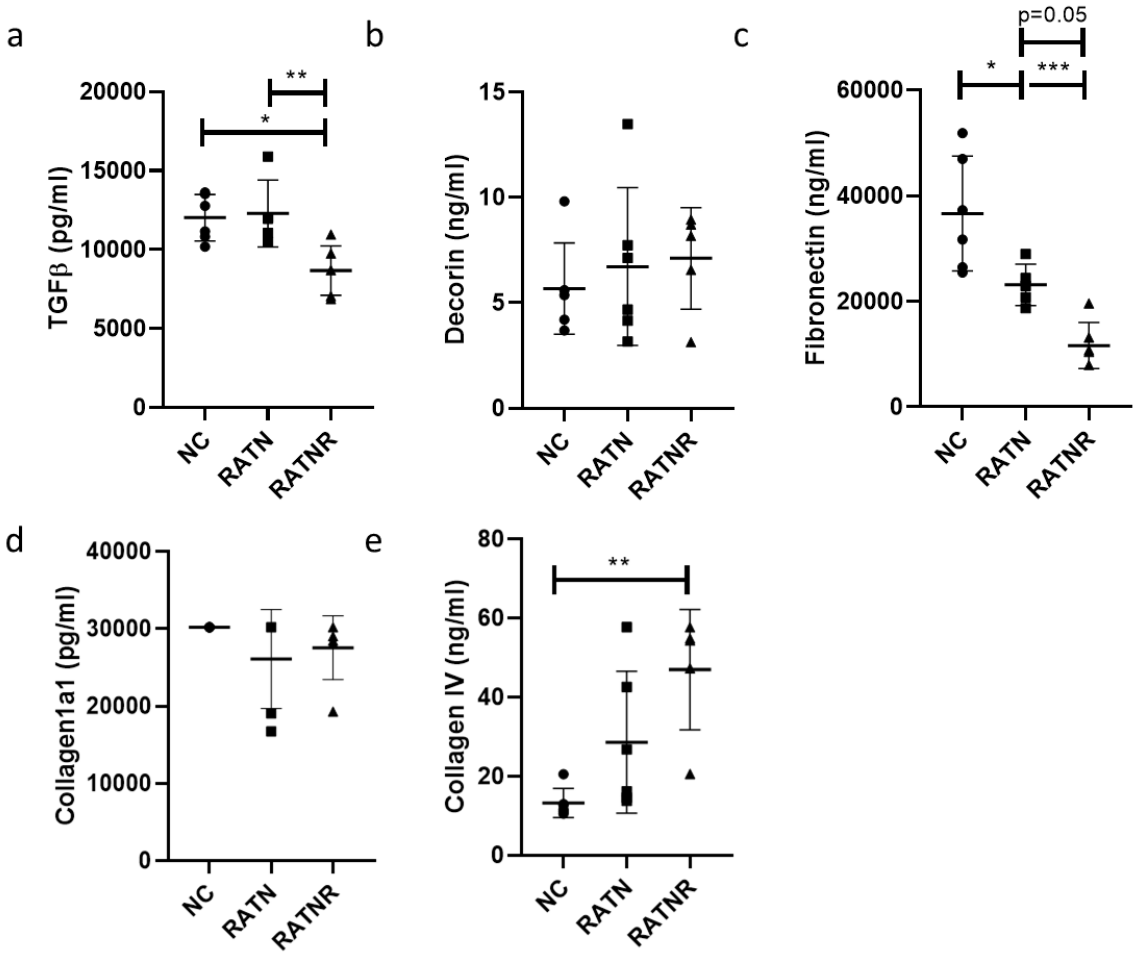


*Figure 5.8: Triple culture supernatant extracellular matrix factor concentration comparing healthy (NC), RA treatment naïve (RATN), and RA treatment non-responding (RATNR) patients. a) TGF-β; b) decorin; c) fibronectin; d) collagen 1a1; e) collagen IV. Statistical analysis: one-way ANOVA (parametric: a, b, c, d) or Kruskal-Wallis test (non-parametric: e). * = p<0.05; ** = p<0.01; *** = p<0.001. n=6 per group. Data represented as mean ± SD. TGF-β = transforming growth factor-β; BMP-7 = bone morphogenetic protein 7.*

Table 5.1 presents the effects of BMP-7 on actual concentrations in the NC by providing the placebo and the BMP-7 data. Here, the addition of BMP-7 decreased the concentration of TGF-β and fibronectin significantly when the triple culture was cultured in NC serum, suggesting that its main effect is exerted on the fibroblasts at the concentration of 750 ng/ml.

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*Table 5.1: Comparison of the concentrations of cytokines and growth factors in the triple co-culture exposed to NC patient serum after being treated with placebo or BMP-7. Data represented as mean ± SD. * = p<0.05. n=6 per group.*

| | Non-arthritic control (NC) | |
|---|---|---|
| Treatment | Placebo | BMP-7 |
| TNF-α (pg/ml) | 18.52 ± 2.0 | 17.15 ± 1.5 |
| IL-6 (pg/ml) | 5561 ± 1122.8 | 5710 ± 1227.4 |
| HGF (pg/ml) | 201.6 ± 25.8 | 189.4 ± 20.7 |
| Follistatin (pg/ml) | 651.3 ± 79.3 | 625.2 ± 84.9 |
| Myostatin (pg/ml) | 1478 ± 631.0 | 1381 ± 319.5 |
| TGF-β (pg/ml) | 12017 ± 1478 | 10423 ± 1198 * |
| Decorin (ng/ml) | 5.68 ± 2.17 | 5.90 ± 2.38 |
| Fibronectin (ng/ml) | 36633 ± 10883 | 19408 ± 7173 * |
| Collagen IV (ng/ml) | 13.20 ± 3.71 | 21.89 ± 9.30 |

To compare how the culture groups responded differently in the presence of BMP-7, data are presented as a percentage of the placebo condition for each patient, in order to normalise data and maximise measurable effect size. In line with the generally low culture inflammatory cytokine responses, neither TNF-α, nor IL-6 secretion was significantly affected by BMP-7 treatment (Figure 5.9a,b). Follistatin concentration, which was significantly decreased as result of RA serum exposure in the placebo conditions, demonstrated a significant increase in concentration when compared to NC, in the presence of BMP-7 in (Figure 5.9d). A similar normalisation effect of BMP-7 was seen for several other parameters assessed, for example with myostatin (Figure 5.9e) and TGF-β concentration (Figure 5.9f), which both exhibited

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significant increases in the RATNR group and both RA groups respectively when compared to the NC group in the presence of BMP-7. There were no significant differences between the patient groups for both decorin (Figure 5.9g) or fibronectin (Figure 5.9h). However, for collagen IV concentration, which was demonstrated to increase in RA (Figure 5.8e), was significantly decreased in the RATNR group compared to the NC as a result of BMP-7 treatment (Figure 5.9i).

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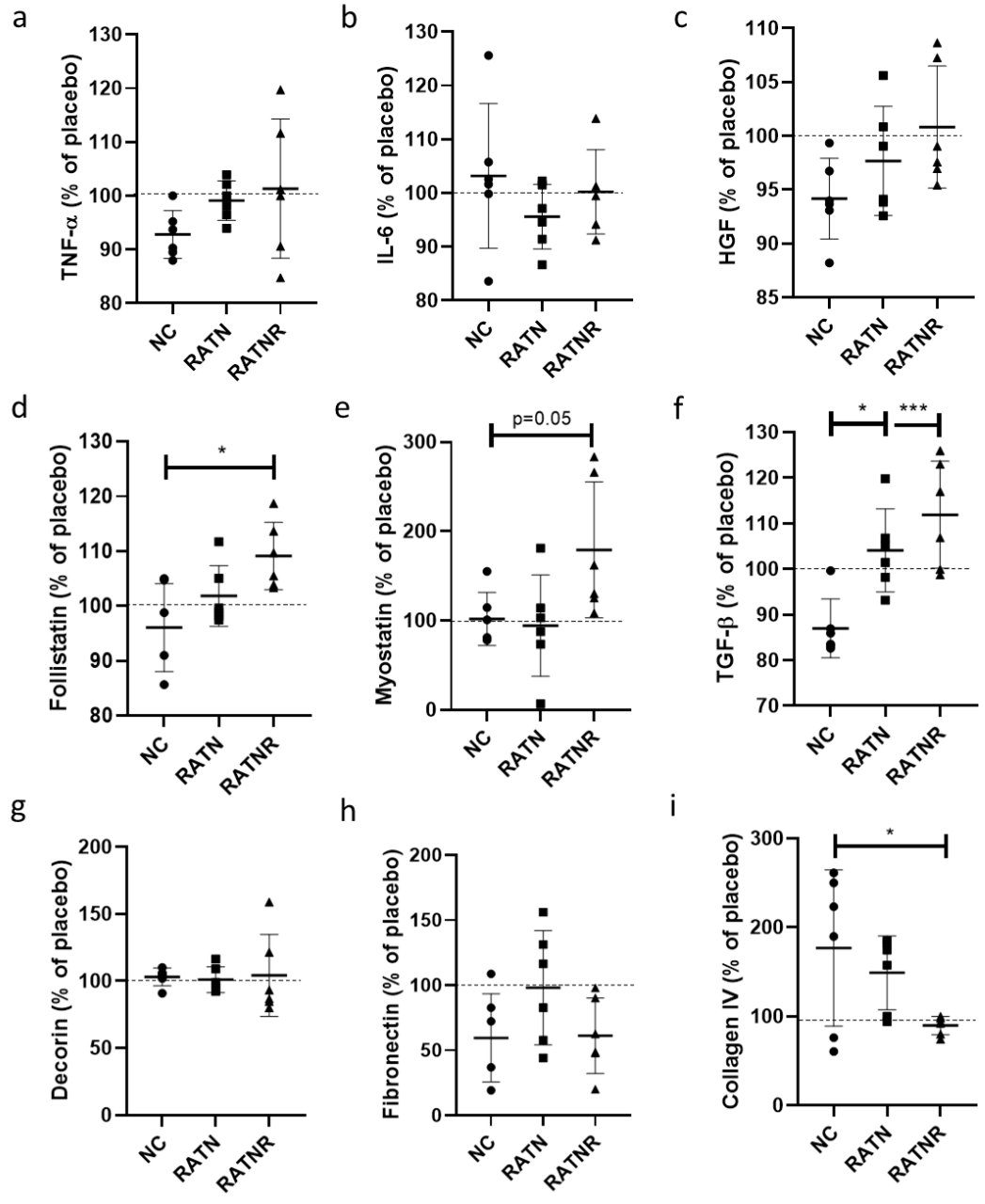


*Figure 5.9: Cellular responses after treatment with BMP-7 expressed as percentage of response in placebo condition, as assessed in triple culture supernatant of a fibroblast, myoblast and M1 macrophage mixed culture exposed to serum of healthy (NC), RA treatment naïve (RATN), and RA treatment non-responding (RATNR) patients. a) TNF-α; b) IL-6; c) HGF; d) follistatin; e) myostatin; f) TGF-β; g) decorin; h) fibronectin; i) collagen IV. Statistical analysis: one-way ANOVA (parametric: a-e, g-i) or Kruskal-Wallis test (non-parametric: f). * = p<0.05; ** = p<0.01; *** = p<0.001. n=6 per group. Data represented as mean ± SD. TNF-α = tumor necrosis factor-α; IL*

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*= interleukin; HGF = hepatocyte growth factor; TGF-β = transforming growth factor-β; BMP-7 = bone morphogenetic protein 7.*

### 5.5 Discussion

Using a novel triple culture technique to simulate the skeletal muscle niche, current data contribute significantly to our understanding of disease mechanisms in RA and RA treatment failure. Furthermore, the efficacy of BMP-7 as potential treatment modality is highlighted.

Before interpreting the effect of a normal mixed culture to patient and control serum, it is necessary to consider differences between the experimental group plasma profiles. In terms of plasma characterisation, cytokine profiles demonstrated the expected inter-individual variability in all groups. Nevertheless, both plasma TNF-α and IL-1β tended to be more variable between individuals - and somewhat higher than those of controls - in both RA groups, which is in line with their known role in the pathogenesis of RA (Kay and Calabrese, 2004; Vasanthi, Nalini and Rajasekhar, 2007). Similarly, the barely detectable levels of IL-1Ra in RATN patients, is again in line with literature correlating this profile with RA disease development (Kay and Calabrese, 2004; Ismail *et al.*, 2016). Various treatment options result in the downstream increase in IL-1Ra (Cutolo *et al.*, 2001; Mizwicki *et al.*, 2012; Schuster *et al.*, 2020), in line with the results demonstrated in the RATNR group. Given its major role as myokine (Pedersen and Febbraio, 2012), IL-6 was also assessed in the current study. However, given the high variability of this parameter even in the control group, interpretation of IL-6 data in isolation was not informative in the current context. However, when correlated with IL-10 levels, important cytokine dysregulation became evident. Under healthy circumstances, IL-6 results in the upregulation of IL-10 (Jin, Han and Yu, 2013; Smith and Swart, 2016). However, this relationship appears to be dysregulated in the RATNR group, where a negative correlation was observed. This relative failure to upregulate IL-10 in response to IL-6 is in line with our earlier suggestion of a failure of RA macrophages to switch to the anti-inflammatory M2c phenotype, which is responsible for IL-10 release (Ollewagen, Myburgh, *et al.*, 2021) as well as our recent study in CIA rats, which demonstrated decreased IL-10 concentration in muscle to be a robust marker for rheumatoid

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cachexia in this model (Ollewagen, Powrie, *et al.*, 2021). This aspect should be elucidated further in longitudinal studies in human RA patients, to fully evaluate the potential of IL-10 as biomarker of disease progression and risk of muscle cachexia in particular. Taken together, the plasma profile data confirm that the samples used as stimulus in the triple cultures, were indeed representative of the expected control and RA cytokine profiles.

Turning attention to the triple culture data, cell growth patterns between the 3 serum conditions differed significantly. RATNR serum-conditioned media resulted in increased cellular growth rates when compared to the other conditions. However, despite the abundance of cells present in RATNR serum exposed conditions, the myoblasts did not attempt to align and begin the differentiation and fusion process. Additionally, based on their appearance, the macrophages in this condition exhibited a greater extent of activation. These effects were likely the result of various growth factors and cytokines present in the serum stimulating the proliferation of myoblasts and fibroblasts. This aligns with the data observed in the rodent CIA model where increased cellular presence was observed, while the ratio between the cells did not differ (Ollewagen, Powrie, *et al.*, 2021). While distinguishing between the cells in single culture was possible due to the low desmin/high fibronectin expression in fibroblasts and low fibronectin/high desmin expression in myoblasts, myoblasts and fibroblasts interacting in co-culture and stimulated by the growth factors in the serum made identification impossible as expression of these proteins in both cell types increased (Seidel *et al.*, 2006; Santiago *et al.*, 2010; Chaturvedi *et al.*, 2015). Therefore, the determination of individual cell type counts was not possible. Perhaps labelling cells with GFP prior to co-culture could clarify specific cell distribution in future studies. However, the most valuable information to consider in this context pertains to the processes at play, and therefore the measurement of the nett secretory products in the muscle niche. Additionally, assessment of these molecular changes is limited in rheumatoid cachexia research.

Our interpretation that the relatively pro-inflammatory cytokine profile in the RA patient plasma may have contributed to the enhanced cellular growth demonstrated in the co-culture, is in line with literature reporting that TNF-α, IL-1β and IL-6 induce myoblast proliferation (Alvarez *et al.*, 2020). Furthermore, the negative IL-6/IL-10 correlation in the RATNR plasma contributes to a dysregulated muscle growth

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pattern - the magnitude of secreted IL-6 in the triple culture is indicative of a muscle response, rather than an inflammatory response (Pedersen, Steensberg and Schjerling, 2001). Different concentrations of IL-6 have differential effects on myoblasts - low concentrations result in proliferation and high concentrations result in differentiation (Steyn *et al.*, 2019). Despite a higher plasma IL-6 concentration, the relatively lower secreted IL-6 in RATNR co-culture conditions may be indicative of an altered response to IL-6 thereby affecting the ability to differentiate. Extensive myoblast/satellite cell proliferation and inhibited differentiation is proposed in chronic inflammatory conditions and RA due to the altered inflammatory profile (Ollewagen, Myburgh, *et al.*, 2021).

In terms of myoblast response, current data illustrates significant dysregulation of normal muscle maintenance signalling in RA. Normally, release of nitric oxide (NO) by active skeletal muscle and macrophages would lead to the release of HGF and subsequent satellite cell activation (Lin and Scott, 2007; Ceafalan, Popescu and Hinescu, 2014; Rigamonti *et al.*, 2014), while IL-6 promotes the production of HGF (Coudriet *et al.*, 2010). NO also induces the expression of follistatin (Lin and Scott, 2007; Ceafalan, Popescu and Hinescu, 2014) to contribute to hypertrophy through satellite cell activation, proliferation and differentiation (Gilson *et al.*, 2009; Zhu *et al.*, 2011) and in rodents demonstrated improved regeneration and reduced fibrosis. Another factor contributing to myogenesis, TGF-β, is a multifunctional cytokine exhibiting various effects on different cell types. (Henningsen *et al.*, 2010; Delaney *et al.*, 2017). The aberrant downregulated tendency of IL-6, HGF, follistatin, and TGF-β in the RATNR serum exposed conditions suggest that rheumatoid cachexia may, at least in part, result from a balance-shift to favour proliferation of muscle tissue, while failing to allow for sufficient differentiation of newly formed cells.

The extracellular environment is another major contributor to signalling, either to enhance or limit tissue maintenance processes. The muscle fibres reside in a scaffold composed of various structural components, referred to as the extracellular matrix (ECM). The ECM is vital in numerous physiological processes in the regulation of muscle development, growth and repair through its interactions with various cell types, including fibroblasts and immune cells (Csapo, Gumpenberger and Wessner, 2020). For example, TGF-β is sequestered to the ECM to upregulate components vital to the structure of the ECM, providing stability and a site for protein

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interactions (Henningsen *et al.*, 2010). However, TGF-β also contributes to fibrosis by stimulating excessive proliferation of fibroblasts and secretion of ECM components, along with inhibition of degradation enzymes (Kim and Lee, 2017). Of the collagens present in the ECM, type I and III are most abundant in the ECM, whereas type IV provides a network structure to form the basal lamina (Csapo, Gumpenberger and Wessner, 2020). Fibronectin, another ECM component, also influences the balance between differentiation and self-renewal, ultimately maintaining the regenerative capacity of the muscle (Bentzinger *et al.*, 2013; Csapo, Gumpenberger and Wessner, 2020). Both myoblasts and fibroblasts are involved in the production of collagens and fibronectin (Kühl, Timpl and von der Mark, 1982; Zou *et al.*, 2008). Here, the reduced TGF-β demonstrated in the RATNR serum exposed conditions coincides with the same decreasing trend observed for collagen 1a1 and fibronectin, ultimately indicating impaired structure and organisation. Reduced fibronectin is also already observed in the RATN group, indicating impaired ECM early in disease development (Farrow *et al.*, 2021). However, collagen IV is increased in the RATNR conditions. Extensive myoblast proliferation may contribute to the increased collagen IV secretion to form the basal lamina. However, altered basal lamina production as a result of increased collagen IV in aged muscle influences the regulation of satellite cell division resulting in impaired satellite cell numbers (Csapo, Gumpenberger and Wessner, 2020). This sub-optimal organisation may be one of the causes of the functional deficits observed in RA patients (Yamada *et al.*, 2009; Uutela, Kautiainen and Häkkinen, 2018). These effects of RA on signalling assessed in vitro is graphically presented in Figure 5.10.

In terms of intervention, in line with the beneficial effects elucidated for BMP-7 in myoblast, fibroblast and macrophage monocultures, treatment of the co-culture with BMP-7 largely reversed the undesired cellular responses observed after exposure to RA serum, and more specifically, normalised the responses of cells treated with RATNR serum, improving deficits in muscle growth markers and ECM markers, without increasing the deposition of fibronectin, indicating a beneficial role of this treatment.

Furthermore, in the context of macrophage phenotype specifically, pilot data indicated that treatment of primary M1 macrophages with 500 and 750 ng/ml of BMP-7 for 48 hours resulted in significantly increased polarisation to M2c

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macrophages (Appendix E). This is in line with literature reporting similar effects for BMP-7 in non-RA models of inflammation in cells and rodents (Singla *et al.*, 2012; Singla, Singla and Wang, 2016). In the triple co-culture model, this benefit of BMP-7 was not evident from the measured cytokine profile. However, this may have been the effect of relative overgrowth of myoblasts and fibroblasts while macrophages don't proliferate. Although increasing the proportion of macrophages in this culture even further may allow for a more representative picture of the macrophage signalling, the beneficial effect of BMP-7 on overall signalling support a shift to an anti-inflammatory phenotype. Given the known lingering presence of M1 and M2b macrophages in RA (Ollewagen, Myburgh, *et al.*, 2021), current data in single and co-culture warrants further investigation of BMP-7 as a treatment modality in RA.

When addressing muscle growth changes, fibre hypertrophy, as observed following injection of BMP-7 vectors into healthy mouse muscle (Winbanks *et al.*, 2013), would be beneficial to RA patients. Preliminary data indicated that when treating primary myoblasts with different dosages of BMP-7, 750 ng/ml resulted in a greater myoblast size (Appendix E). The reversal of both HGF and follistatin effects in the RATNR serum exposed cultures highlights a potentially beneficial effect. One conflicting factor that may inhibit this effect is the increased myostatin levels in the RATNR serum exposed conditions, due to its ability to bind to the BMP-7 receptors (Rebbapragada *et al.*, 2003), thereafter inhibiting its effects. However, without competitive binding data, it is not possible to make firm conclusions on this.

Lastly, based on the presence of significant fibrosis in rheumatoid cachexia in the CIA model (Oyenihi *et al.*, 2019) and the above findings of impaired ECM formation in RATNR serum exposed co-cultures, the normalising action of BMP-7 on ECM formation warrants further investigation. As observed in NC serum exposed conditions, BMP-7 has anti-fibrotic effects through the inhibition of TGF-β (McVicker and Bennett, 2017) and limitation of ECM accumulation. While a seemingly opposing effect is observed for BMP-7 in the RATNR group, the outcome observed was still a return towards normal, via improving the overall structure of the ECM and improving the overall outcome. These effects of BMP-7 treatment on RA patient-exposed co-culture are again presented in Figure 5.10.

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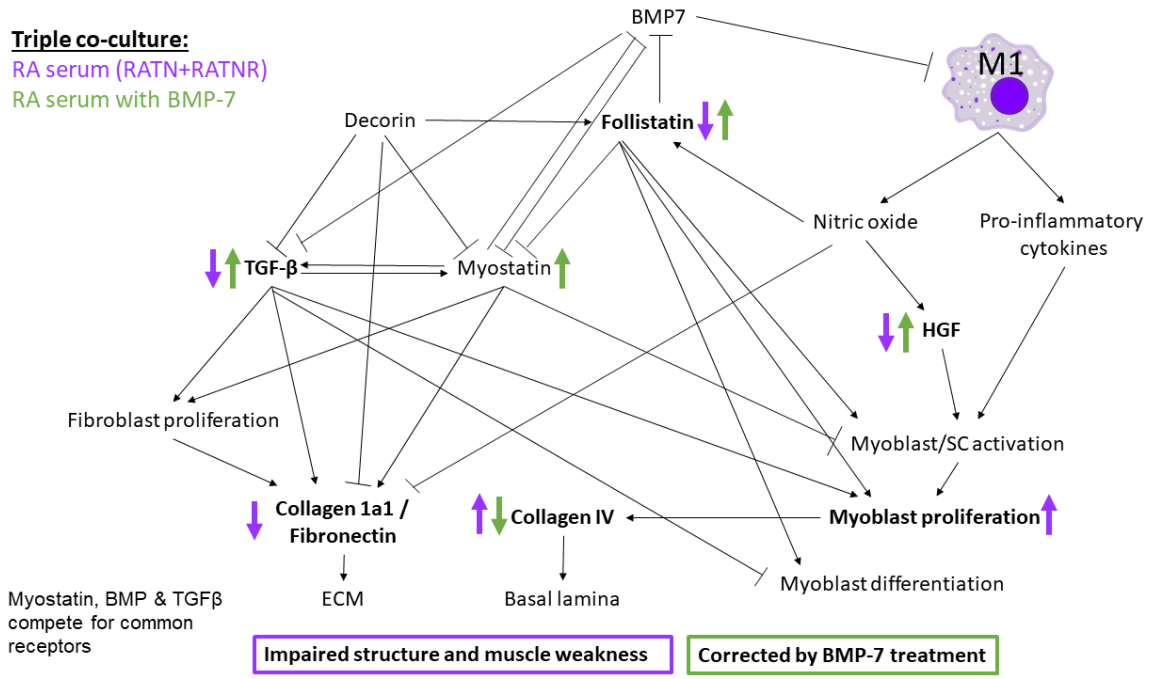


*Figure 5.10: Summary of pathways implicated in the triple co-culture experiment as proposed by literature. Purple arrows indicate the changes occurring as a result of RA patient serum-conditioned media. Green arrows indicated the changes occurring as a result of BMP-7 treatment. BMP-7 = bone morphogenetic protein-7; HGF = hepatocyte growth factor; TGF-β = transforming growth factor-β*

## 5.6 Conclusion

Current data demonstrated plasma cytokine differences indicative of healthy controls and RA patients, with a more severe outcome in treatment non-responsive patients, which may be either due to treatment resistance itself, or a longer duration of disease progression when compared to treatment naïve patients. One of the key factors in the RATNR group is the dysregulation between plasma IL-6 and IL-10 which may impact the downstream muscle effects. Through the use of a novel, primary triple co-culture method, the RATNR serum exposed group exhibited extensive capacity for cell growth, despite downregulated HGF and follistatin, and suboptimal ECM organisation when compared to controls. BMP-7 treatment showed beneficial results by reversing the aberrant tendencies observed in the cultures exposed to RA serum.

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On a practical note, current data illustrates our novel co-culture model to be an accurate simulation of signalling events in RA and thus potentially a powerful tool in understanding rheumatoid cachexia and developing patient-specific treatment strategies.

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# Chapter 6: Synthesis

Despite rheumatoid cachexia occurring in a large number of patients and reducing their quality of life, investigation into the actual mechanisms at play in the development of the disease is a relatively neglected area of focus in research. The fact that a number of current RA treatment options contribute to the muscle wasting, highlights the priority for the research presented in this dissertation.

Firstly, in this dissertation, the literature was comprehensively reviewed to construct a profile of the altered muscle structure in rheumatoid arthritis, as well the nature of inflammatory dysregulation and its contribution to a cachexic cellular environment. Review of the literature revealed a relative lack of RA-specific information on muscle wasting, so that information from non-RA models of inflammation had to be extrapolated to develop a working model for signalling in RA. This review (Chapter 2) was published in a high-ranking journal, illustrating the need for this information in the field. Considering the limited research specific to rheumatoid cachexia, more investigation was required to determine the nature of atrophy and relevant cell interactions and the molecular role players in the development of atrophy, to develop accurate pre-clinical tools with which to develop and assess treatment strategies. The descriptive studies in the rodent collagen-induced arthritis (CIA) model were conducted first.

The majority of muscle research in humans is conducted on the *vastus lateralis*, a muscle composed of a mixed fibre type. However, considering that muscles vary in the composition of fibres, the aim of the first rodent study was to assess muscle morphology and ultrastructural changes in four hindlimb muscles, along with the redox changes in a rodent collagen-induced arthritis model (Chapter 3). The *gastrocnemius, extensor digitorum longus* (EDL) and *soleus* demonstrated a lower muscle mass, whereas the *vastus lateralis* did not. Nonetheless, signs of deterioration indicative of rheumatoid cachexia were present in all four muscle groups. All muscle groups had significantly reduced fibre CSA and significantly increased collagen deposition with increased tissue levels of ROS. The muscle group most affected was the *gastrocnemius* muscle, which also exhibited detrimental changes in lipid peroxidation and antioxidant activity. Overall, despite the protocol

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limitation of not being a longitudinal design, instead a cross-sectional assessment carried out at a relatively early time point in the development of this chronic disease, results proved this to be a successful model for investigating rheumatoid cachexia. The manuscript prepared from this data was selected for publication in a special issue of Oxidative Medicine and Cellular Longevity (2019, impact factor 6.543) entitled “Effects of redox disturbances on motility, contractility and muscle tissue pathogenesis”.

Given the complexity of the disease, it is of course near impossible to cover all aspects in every experiment. For example, considering the definition of rheumatoid cachexia as a loss of muscle mass with or without an increase in fat mass (Roubenoff *et al.*, 1994), an investigation into the changes in intramuscular fat deposition in the different muscle groups may have added to the picture of ultrastructural changes occurring in arthritis-affected muscle. Additionally, a potential criticism may be that the reduction in muscle mass in the CIA model may have been due to reduced movement as a result of pain to the affected joints. Although no noticeable changes in gait or activity status were observed, this was not quantified. Although suitable equipment was not available at the time, activity tracking in future studies may provide further valuable insight.

Nevertheless, having established the CIA model as accurate simulation of arthritis-associated muscle wasting, the aim of the second rodent study (Chapter 4) was to investigate the changes in cell types, cytokines and growth factors in the CIA model to gain an accurate understanding of the signalling mechanisms at play in the development of rheumatoid cachexia. A novel aspect of this study was the assessment of the cellular profile in arthritis-affected skeletal muscle tissue, as well as demonstrating the first comprehensive assessment of muscle regenerative capacity and intramuscular cytokine profile in CIA. A crucial outcome of this study was highlighting the importance of considering fibre size and number when assessing cell number, cytokines and growth factors to get an accurate view of the changes relative to each fibre. Data demonstrated that the two most instrumental cytokines in the development of rheumatoid cachexia are the increased TNF-α and reduced IL-10, which is consistent with a lingering M1 macrophage phenotype. This study importantly indicated that despite the persistent pro-inflammatory state, the cachexic muscle seemed to retain at least some capacity for regeneration.

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Specifically, proliferation markers were consistently upregulated, while differentiation marker expression was somewhat contradictory, suggesting at least some extent of dysregulation, particularly in the progression of regeneration. As result, the overall regenerative capacity was not sufficient to overcome the extent of atrophy caused by the unresolved inflammation. This reiterated the fact that no secondary outcome of a chronic inflammatory disease can be adequately addressed unless the resolution of chronic inflammation remains a primary focus.

In terms of limitations, this study – which was focused on the muscle regenerative capacity in arthritis-affected muscle - may have benefitted firstly from inclusion of assessments of the macrophage phenotype profile to confirm incomplete phenotype conversion as driving force in the muscle niche. Secondly, given the focus on muscle-specific markers of regeneration rather than intercellular communication networks, parameters reflecting mechanisms of fibrosis and how this might have affected the muscle was limited to TGF-β. Furthermore, considering the reported presence of fibro-adipogenic progenitor cells (FAPs) in RA (Hogarth *et al.*, 2019; Giuliani, Rosina and Reggio, 2021) - and their contribution to adipose and ECM deposition – characterisation of fibroblasts/FAPs would have added further valuable information on their role in muscle wasting disorders.

Finally, to further understand the interactive cellular signalling from multiple cellular role players in rheumatoid cachexia, a novel triple co-culture method was developed using primary human myoblasts, fibroblasts and macrophages, in the presence of RA patient and healthy participant serum (Chapter 5). Here, two groups of RA patients were assessed, namely treatment naïve and treatment non-responding patients. Although plasma parameters displayed the normal variability and sample size had to be limited because of the high cost of reagents and assays, it was possible to confirm that patient plasma profiles were in line with a relatively more pro-inflammatory state than in the control plasma. More specifically, both RA groups exhibited dysregulated IL-6/IL-10 plasma correlation, which was in line with the lower IL-10 levels measured in the CIA rodents.

One of the novel aspects of the triple co-culture model was that the ratio of myoblasts to fibroblasts to macrophages was determined based on the relative presence of these cells in the CIA rodent muscle, thereby simulating rheumatoid

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cachexia more closely. Higher cell confluence demonstrated a significantly greater cell proliferation rate in the RATNR serum-exposed cultures when compared to those stimulated with serum from healthy controls. This is in agreement with the increased proliferation markers seen in the rodent study, confirming the accuracy of the model in simulating *in vivo* processes. In addition, perhaps due to the higher level of standardisation possible *in vitro*, coupled with use of serum from patients at a later phase in disease progression, the *in vitro* data more clearly illustrated the effect of arthritis on muscle cell regeneration. The assessment of muscle-specific proliferation and growth markers in the supernatant of the triple co-culture demonstrated downregulation in both RA groups, but significantly so only in the RATNR group, indicating dysregulation of the muscle progenitor cells. In addition to this, the RATNR group demonstrated decreased collagen 1a1 and fibronectin, and increased collagen IV, indicative of suboptimal ECM organisation, which would tie into the functional deficits observed in clinical assessments of RA patients.

An assessment of potential therapeutic intervention and the sensitivity of the model to reflect potential therapeutic effects, was tested by determining the response of co-cultured cells to BMP-7 treatment. Treatment reduced markers of the fibrotic process in the NC group, whereas BMP-7 treatment in the RATNR group seemed to correct/limit the RA-associated effects observed. This model thus not only shows promise as tool with which to elucidate the mechanisms behind rheumatoid cachexia, but also as a model to develop treatment strategies for the disease as a whole, and for monitoring individual patient-based outcomes. For example, using this model to determine which molecular markers and thus, which cell types are most affected by patients' sera could give insight into treatment targets. This model could assess the individual patient-specific responses, and thereafter investigate potential patient-specific treatment targets or the effect of new RA treatments' effect on the cells relevant to this secondary disease.

Despite the promise of the model described, several limitations need to be solved. Due to the high cost already mentioned, this study was limited to an assessment at the protocol endpoint (96 hours). Given a larger budget, added assessments at 48 hours (prior to BMP-7 treatment) may have potentially demonstrated different responses of cells at lower confluence, specifically in the RATNR group, where the rapid cellular proliferation and thus high confluence of cultures, may have down-

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regulated some pro-regeneration signalling. However, given the fact that the corrective action of BMP-7 was detectable, I am confident that the choice of time-point and the relatively higher confluence of RATNR serum-exposed cultures, do not limit the accuracy or applicability of presented data.

Due to the lack of markers specific enough to differentiate between co-cultured primary myoblasts and fibroblasts, the specific cell types present in the differentially conditioned co-cultures was not assessed. Therefore, it is still unknown which cell type (myoblast or fibroblast) was the most proliferative in the different triple co-culture groups. Additionally, characterisation of the macrophage phenotype most predominantly present in the different groups would be beneficial in confirming the responses predicted by the protein outcomes, and which phenotype is persistent to confirm the literature predictions.

In conclusion, this dissertation, by inclusion of studies ranging from rodent skeletal muscle to RA patient plasma, and finally, to primary triple cell co-culture, has provided significant insight into the cellular and molecular role players in the development of rheumatoid cachexia. It is clear that the inflammation associated with RA, results in significant muscle wasting and fibrosis in various muscle groups, independent of fibre-type composition. Despite the muscle remaining capable of regeneration, progression of this process is severely hampered due to the persistent inflammation. However, extensive proliferation of the satellite cells and myoblasts could eventually lead to a reduction in the satellite cell pool, resulting in impaired regenerative capacity in the later stages of RA. The persistent inflammation results in muscle dysregulation and altered ECM formation, confirming the source of impaired structure and function in RA patients. Finally, BMP-7 is highlighted as a factor that could restore or prevent the extent of cachexia observed in RA patients.

Looking forward, I believe that the triple cell co-culture model I have developed – and which has proven to be an accurate and sensitive simulation of the cellular release of cytokines, growth factors and other relevant molecules in RA – should be further exploited to address this very complex and very debilitating disease. This model could be vital in understanding patient-specific outcomes and treatment strategies. In order to make the model even more accurate, introduction of FAPs as additional cell type in relation to intramuscular lipid accumulation could be introduced as a protocol

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modification. The fact that non-diseased cells are used for the triple co-culture, means that it may be standardised using commercial cell lines to reduce costs and improve throughput, although immortalised cells do not fully represent the responses of primary cells. The application of the model could also be expanded to other chronic inflammatory diseases with a muscle wasting outcome, and new therapeutics may be tested more comprehensively before being introduced into *in vivo* models.

In combination with modern and new emerging technologies, parameters assessed can also be expanded. For example, the use of imaging flow cytometry can give better insight into the intracellular/extracellular changes of all three cell types as one could measure protein changes both analytically and microscopically. Analysing plasma-derived exosomes and their microRNA cargo can give more insight into alterations in cell-cell communication and immune biomarkers in RA patients. This method would also be valuable when translated into the triple co-culture model for further assessment of intercellular communication. Proteomic analysis could be used to investigate proteins at a comprehensive scale, both in the supernatant and in the cells themselves, allowing broader assessment of changes resulting from patient serum exposure.

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# Chapter 7: References

Abramowitz, M.K. *et al.* (2018) Skeletal muscle fibrosis is associated with decreased muscle inflammation and weakness in patients with chronic kidney disease, *American Journal of Physiology - Renal Physiology*, 315(6), pp. F1658–F1669. doi:10.1152/ajprenal.00314.2018.

Agrawal, V., Husain, N., Das, S.K. and Bagchi, M. (2003) Muscle involvement in rheumatoid arthritis: clinical and histological characteristics and review of literature, *J Indian Rheumatol Asoc*, 11, pp. 98–103.

Alameddine, H.S. and Morgan, J.E. (2016) Matrix metalloproteinases and tissue inhibitor of metalloproteinases in inflammation and fibrosis of skeletal muscles, *Journal of Neuromuscular Diseases*, 3, pp. 455–473. doi:10.3233/JND-160183.

Allbrook, D. (1981) Skeletal muscle regeneration, *Muscle & Nerve*, 4, pp. 234–245.

Alvarez, A.M., DeOcesano-Pereira, C., Teixeira, C. and Moreira, V. (2020) IL-1β and TNF-α modulation of proliferated and committed myoblasts: IL-6 and COX-2-derived prostaglandins as key actors in the mechanisms involved, *Cells*, 9, pp. 1–17. doi:10.3390/cells9092005.

Alway, S.E., Martyn, J.K., Ouyang, J., Chaudhrai, A. and Murlasits, Z.S. (2003) Id2 expression during apoptosis and satellite cell activation in unloaded and loaded quail skeletal muscles, *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, 284, pp. 540–549. doi:10.1152/ajpregu.00550.2002.

Alway, S.E., Myers, M.J. and Mohamed, J.S. (2014) Regulation of satellite cell function in sarcopenia, *Frontiers in Aging Neuroscience*, 6, pp. 1–15. doi:10.3389/fnagi.2014.00246.

Andonian, B.J. and Huffman, K.M. (2020) Skeletal muscle disease in rheumatoid arthritis: the center of cardiometabolic comorbidities?, *Current Opinions in Rheumatology*, 32(3), pp. 297–306. doi:10.1097/BOR.0000000000000697.

Araki, Y. and Mimura, T. (2016) Inflammation in Rheumatoid Arthritis from the Perspective of the Epigenetic Landscape, *Journal of Immunology Research*, 2016, pp. 1–10.

Stellenbosch University https://scholar.sun.ac.za

Argilés, J.M., López-Soriano, F.J., Toledo, M., Betancourt, A., Serpe, R. and Busquets, S. (2011) The cachexia score (CASCO): A new tool for staging cachectic cancer patients, *Journal of Cachexia, Sarcopenia and Muscle*, 2(2), pp. 87–93. doi:10.1007/s13539-011-0027-5.

Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., Van Rooijen, N., Plonquet, A., Gherardi, R.K. and Chazaud, B. (2007) Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis, *Journal of Experimental Medicine*, 204(5), pp. 1057–1069. doi:10.1084/jem.20070075.

Asquith, D.L., Miller, A.M., McInnes, I.B. and Liew, F.Y. (2009) Animal models of rheumatoid arthritis, *European Journal of Immunology*, 39(8), pp. 2040–2044. doi:10.1002/eji.200939578.

Ato, S., Kido, K., Sato, K. and Fujita, S. (2019) Type 2 diabetes causes skeletal muscle atrophy but does not impair resistance training-mediated myonuclear accretion and muscle mass gain in rats, *Experimental Physiology*, 104, pp. 1518–1531. doi:10.1113/EP087585.

Baker, J.F., Feldt, J.M. Von, Mostoufi-moab, S., Kim, W., Taratuta, E. and Leonard, M.B. (2015) Insulin-like growth factor 1 and adiponectin and associations with muscle deficits, disease characteristics, and treatments in rheumatoid arthritis, *Journal of Rehabilitation Medicine*, 42(11), pp. 2038–2045. doi:10.3899/jrheum.150280.Insulin-like.

Bala, A. (2017) Oxidative stress in inflammatory cells of patient with rheumatoid arthritis: clinical efficacy of dietary antioxidants, *Inflammopharmacology*, 25(6), pp. 595–607. doi:10.1007/s10787-017-0397-1.

Barreiro, E., De La Puente, B., Busquets, S., López-Soriano, F.J., Gea, J. and Argilés, J.M. (2005) Both oxidative and nitrosative stress are associated with muscle wasting in tumour-bearing rats, *FEBS Letters*, 579(7), pp. 1646–1652. doi:10.1016/j.febslet.2005.02.017.

Bearne, L.M., Scott, D.L. and Hurley, M. V. (2002) Exercise can reverse quadriceps sensorimotor dysfunction that is associated with rheumatoid arthritis without exacerbating disease activity, *Rheumatology*, 41(2), pp. 157–166.

Stellenbosch University https://scholar.sun.ac.za

doi:10.1093/rheumatology/41.2.157.

Bencze, M. *et al.* (2012) Proinflammatory macrophages enhance the regenerative capacity of human myoblasts by modifying their kinetics of proliferation and differentiation, *The American Society of Gene & Cell Therapy*, 20(11), pp. 2168–2179. doi:10.1038/mt.2012.189.

Bentzinger, C., Wang, Y., von Maltzahn, J., Soleimani, V., Yin, H. and Rudnicki, M. (2013) Fibronectin regualtes Wnt7a signaling and satellite cell expansion, *Cell Stem Cell*, 12(1), pp. 75–87. doi:10.1016/j.stem.2012.09.015.Fibronectin.

Bersini, S., Gilardi, M., Mora, M., Krol, S., Arrigoni, C., Candrian, C., Zanotti, S. and Moretti, M. (2018) Tackling muscle fibrosis: From molecular mechanisms to next generation engineered models to predict drug delivery, *Advanced Drug Delivery Reviews*, 129, pp. 64–77. doi:10.1016/j.addr.2018.02.009.

Beziere, N. *et al.* (2019) Imaging fibrosis in inflammatory diseases: targeting the exposed extracellular matrix, *Theranostics*, 9(10), pp. 2868–2881. doi:10.7150/thno.28892.

Biferali, B., Proietti, D., Mozzetta, C. and Madaro, L. (2019) Fibro-adipogenic progenitors cross-talk in skeletal muscle: the social network, *Frontiers in Physiology*, 10, pp. 1–10. doi:10.3389/fphys.2019.01074.

Bilberg, A., Ahlmén, M. and Mannerkorpi, K. (2005) Moderately intensive exercise in a temperate pool for patients with rheumatoid arthritis: A randomized controlled study, *Rheumatology*, 44(4), pp. 502–508. doi:10.1093/rheumatology/keh528.

Blair, H.A. and Deeks, E.D. (2017) Abatacept: A review in rheumatoid arthritis, *Drugs*, 77, pp. 1221–1233. doi:10.1007/s40265-017-0775-4.

Bodine, S.C. and Baehr, L.M. (2014) Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and MAFbx/atrogin-1, *American Journal of Physiology - Endocrinology and Metabolism*, 307(6), pp. E469–E484. doi:10.1152/ajpendo.00204.2014.

Bortolini, M., Wright, M.B., Bopst, M. and Balas, B. (2013) Examining the safety of PPAR agonists - current trends and future prospects, *Expert Opinon Drug Safety*, 12(1), pp. 65–79.

Stellenbosch University https://scholar.sun.ac.za

Boutrup, R.J., Farup, J., Vissing, K., Kjaer, M. and Mikkelsen, U.R. (2018) Skeletal muscle stem cell characteristics and myonuclei content in patients with rheumatoid arthritis: a cross-sectional study, *Rheumatology International*, 38, pp. 1031–1041. doi:10.1007/s00296-018-4028-y.

Boyle, D.L. *et al.* (2015) The JAK inhibitor tofacitinib suppresses synovial JAK1-STAT signalling in rheumatoid arthritis, *Annals of the Rheumatic Diseases*, 74, pp. 1311–1316. doi:10.1136/annrheumdis-2014-206028.

Breedveld, F.C., Weisman, M.H., Kavanaugh, A.F., Cohen, S.B., Pavelka, K., van Vollenhoven, R., Sharp, J., Perez, J.L. and Spencer-Green, G.T. (2006) The PREMIER Study: A multicenter, randomized, double-blind clinical trial of combination therapy with adalimumab plus methotrexate versus methotrexate alone or adalimumab alone in patients with early, aggressive rheumatoid arthritis who had not had previo, *Arthritis & Rheumatism*, 54(1), pp. 26–37. doi:10.1002/art.21519.

Brennan, Fionula M and McInnes, I.B. (2008) Evidence that cytokines play a role in rheumatoid arthritis, *The Journal of Clinical Investigation*, 118(11), pp. 3537–3545. doi:10.1172/JCI36389.in.

Brennan, Fionula M. and McInnes, I.B. (2008) Evidence that cytokines play a role in rheumatoid arthritis, *Journal of Clinical Investigation*, 118(11), pp. 3537–3545. doi:10.1172/JCI36389.

Brink, M., Lundquist, A., Alexeyenko, A., Lejon, K. and Rantapää-Dahlqvist, S. (2019) Protein profiling and network enrichment analysis in individuals before and after the onset of rheumatoid arthritis, *Arthritis Research & Therapy*, 21(288), pp. 1–14.

Ten Broek, R.W., Grefte, S. and Von Den Hoff, J.W. (2010) Regulatory factors and cell populations involved in skeletal muscle regeneration, *Journal of Cellular Physiology*, 224(1), pp. 7–16. doi:10.1002/jcp.22127.

Brorsson, S., Hilliges, M., Sollerman, C. and Nilsdotter, A. (2009) A six-week hand exercise programme improves strength and hand function in patients with rheumatoid arthritis, *Journal of Rehabilitation Medicine*, 41(5), pp. 338–342. doi:10.2340/16501977-0334.

Stellenbosch University https://scholar.sun.ac.za

Caillaud, C., Py, G., Eydoux, N., Legros, P., Prefaut, C. and Mercier, J. (1999) Antioxidants and mitochondrial respiration in lung, diaphragm, and locomotor muscles: effect of exercise, *Free Radical Biology & Medicine*, 26, pp. 1292–1299.

Cao, Y., Nimptsch, K., Shui, I.M., Platz, E.A., Wu, K., Pollak, M.N., Kenfield, S.A., Stampfer, M.J. and Giovannucci, E.L. (2015) Prediagnostic plasma IGFBP-1, IGF-1 and risk of prostate cancer, *International Journal of Cancer*, 136, pp. 2418–2426. doi:10.1002/ijc.29295.

Cappellari, O., Mantuano, P. and De Luca, A. (2020) 'The Social Network' and muscular dystrophies: The lesson learnt about the niche environment as a target for therapeutic strategies, *Cells*, 9(7), pp. 1–28. doi:10.3390/cells9071659.

Castillero, E., Martín, A.I., López-Menduiña, M., Granado, M., Villanúa, M.Á. and López-Calderón, A. (2009) IGF-I system, atrogenes and myogenic regulatory factors in arthritis induced muscle wasting, *Molecular and Cellular Endocrinology*, 309(1–2), pp. 8–16. doi:10.1016/j.mce.2009.05.017.

Castillero, E., Nieto-Bona, M.P., Fernández-Galaz, C., Martín, A.I., López-Menduiña, M., Granado, M., Villanúa, M.A. and López-Calderón, A. (2011) Fenofibrate, a PPARα agonist, decreases atrogenes and myostatin expression and improves arthritis-induced skeletal muscle atrophy, *American Journal of Physiology-Endocrinology and Metabolism*, 300(5), pp. E790–E799. doi:10.1152/ajpendo.00590.2010.

Ceafalan, L.C., Popescu, B.O. and Hinescu, M.E. (2014) Cellular players in skeletal muscle regeneration, *BioMed Research International*, 2014, pp. 1–21. doi:10.1155/2014/957014.

Ceyhan Dogan, S., Hizmetli, S., Hayta, E., Kaptanoglu, E., Erselcan, T. and Guler, E. (2015) Sarcopenia in women with rheumatoid arthritis, *European Journal of Rheumatology*, 2(2), pp. 57–61. doi:10.5152/eurjrheum.2015.0038.

Chapman, M.A., Meza, R. and Lieber, R.L. (2016) Skeletal muscle fibroblasts in health and disease, *Differentiation*, 92(3), pp. 108–115. doi:10.1016/j.diff.2016.05.007.Skeletal.

Chaturvedi, V., Dye, D.E., Kinnear, B.F., Van Kuppevelt, T.H., Grounds, M.D. and

Stellenbosch University https://scholar.sun.ac.za

Coombe, D.R. (2015) Interactions between skeletal muscle myoblasts and their extracellular matrix revealed by a serum free culture system, *PLoS ONE*, 10(6), pp. 1–27. doi:10.1371/journal.pone.0127675.

Chazaud, B., Brigitte, M., Yacoub-Youssef, H., Arnold, L., Gherardi, R., Sonnet, C., Lafuste, P. and Chretien, F. (2009) Dual and beneficial roles of macrophages during skeletal muscle regeneration, *Exercise and Sport Sciences Reviews*, 37(1), pp. 18–22. doi:10.1097/JES.0b013e318190ebdb.

Chen, X. and Li, Y. (2009) Role of matrix metalloproteinases in skeletal muscle: migration, differentiation, regeneration and fibrosis., *Cell Adhesion & Migration*, 3(4), pp. 337–341. doi:10.4161/cam.3.4.9338.

Choy, E.H.S. and Panayi, G.S. (2001) Cytokine pathways and joint inflammation in rheumatoid arthritis, *Mechanisms of Disease*, 344(12), pp. 907–916.

Chu, C.Q., Field, M., Feldmann, M. and Maini, R.N. (1991) Localization of Tumor Necrosis Factor α in Synovial Tissues and at the Cartilage–Pannus Junction in Patients With Rheumatoid Arthritis, *Arthritis & Rheumatism*, 34(9), pp. 1125–1132. doi:10.1002/art.1780340908.

Chung, E., Mo, H., Wang, S., Zu, Y., Elfakhani, M., Rios, S.R., Chyu, M.C., Yang, R. Sen and Shen, C.L. (2018) Potential roles of vitamin E in age-related changes in skeletal muscle health, *Nutrition Research*, 49, pp. 23–36. doi:10.1016/j.nutres.2017.09.005.

Cooper, S.T., Maxwell, A.L., Kizana, E., Ghoddusi, M., Hardeman, E.C., Alexander, I.E., Allen, D.G. and North, K.N. (2004) C2C12 co-culture on a fibroblast substratum enables sustained survival of contractile, highly differentiated myotubes with peripheral nuclei and adult fast myosin expression, *Cell Motility and the Cytoskeleton*, 58(3), pp. 200–211. doi:10.1002/cm.20010.

Costamagna, D., Costelli, P., Sampaolesi, M. and Penna, F. (2015) Role of inflammation in muscle homeostasis and myogenesis, *Mediators of Inflammation*, 2015, pp. 1–14.

Coudriet, G.M., He, J., Trucco, M., Mars, W.M. and Piganelli, J.D. (2010) Hepatocyte growth factor modulates interleukin-6 production in bone marrow derived

Stellenbosch University https://scholar.sun.ac.za

macrophages: Implications for inflammatory mediated diseases, *PLoS ONE*, 5(11), pp. 1–7. doi:10.1371/journal.pone.0015384.

Cronstein, B.N. (2005) Low-dose methotrexate: a mainstay in the treatment of rheumatoid arthritis, *Pharmacological Reviews*, 57(2), pp. 163–172. doi:10.1124/pr.57.2.3.mittently.

Csapo, R., Gumpenberger, M. and Wessner, B. (2020) Skeletal muscle extracellular matrix – what do we know about its composition, regulation, and physiological roles? A narrative review, *Frontiers in Physiology*, 11(March), pp. 1–15. doi:10.3389/fphys.2020.00253.

Curtis, J.R. and Singh, J.A. (2011) Use of biologics in Rheumatoid Arthritis: current and emerging paradigms of care, *Clinical Therapeutics*, 33(6), pp. 679–707. doi:10.1016/j.clinthera.2011.05.044.

Cutolo, M., Sulli, A., Pizzorni, C., Seriolo, B. and STRAUB, R.H. (2001) Anti-inflammatory mechanisms of methotrexate in rheumatoid arthritis, *Annals of the Rheumatic Diseases*, 60(8), pp. 729–735. doi:10.1136/ard.60.8.729.

Daans, M., Lories, R.J.U. and Luyten, F.P. (2008) Dynamic activation of bone morphogenetic protein signaling in collagen-induced arthritis supports their role in joint homeostasis and disease, *Arthritis Research and Therapy*, 10(5), pp. 1–10. doi:10.1186/ar2518.

Day, R. (2002) Adverse reactions to TNF-α inhibitors in rheumatoid arthritis, *Lancet*, 359(9306), pp. 540–541. doi:10.1016/S0140-6736(02)07718-8.

Dayer, J.M. (2002) Interleukin 1 or tumor necrosis factor-α: Which is the real target in rheumatoid arthritis?, *Journal of Rheumatology*, 29(SUPPL. 65), pp. 10–15.

Dayer, J.M. (2003) The pivotal role of interleukin-1 in the clinical manifestations of rheumatoid arthritis, *Rheumatology*, 42(SUPPL. 2), pp. 3–10. doi:10.1093/rheumatology/keg326.

Dayer, J.M., Beutler, B. and Cerami, A.A. (1985) Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts, *Journal of Experimental Medicine*, 162(6), pp. 2163–2168. doi:10.1084/jem.162.6.2163.

Stellenbosch University https://scholar.sun.ac.za

Delaney, K., Kasprzycka, P., Ciemerych, M.A. and Zimowska, M. (2017) The role of TGF-β1 during skeletal muscle regeneration, *Cell Biology International*, 41(7), pp. 706–715. doi:10.1002/cbin.10725.

Díaz, B.B., González, D.A., Gannar, F., Perez, M.C.R. and de Leon, A.C. (2018) Myokines, physical activity, insulin resistance and autoimmune diseases, *Immunology Letters*, 203, pp. 1–5. doi:10.1016/j.imlet.2018.09.002.

Dinarello, C.A. and Wolff, S.M. (1993) The role of interleukin-1 in disease, *The New England Journal of Medicine*, 328(2), pp. 106–113.

Drosos, A.A. (2020) Treatment strategies are more important than drugs in the management of rheumatoid arthritis, *Clinical Rheumatology*, 39, pp. 1363–1368.

Duijnisveld, B.J. *et al.* (2011) Regenerative potential of human muscle stem cells in chronic inflammation, *Arthritis Research & Therapy*, 13, pp. 2–10.

Edwards, J.C.W., Szczepanski, L., Szechinski, J., Filipowicz-Sosnowska, A., Emery, P., Close, D.R., Stevens, R.M. and Shaw, T. (2004) Efficacy of B-cell–targeted therapy with Rituximab in patients with rheumatoid arthritis, *The New England Journal of Medicine*, 350, pp. 2572–2581.

Edwards, J.P., Zhang, X., Frauwirth, K.A. and Mosser, D.M. (2006) Biochemical and functional characterization of three activated macrophage populations, *Journal of Leukocyte Biology*, 80(6), pp. 1298–1307. doi:10.1189/jlb.0406249.Biochemical.

Emery, P., Keystone, E., Tony, H.P., Cantagrel, A., Vollenhoven, R. Van, Sanchez, A., Alecock, E., Lee, J. and Kremer, J. (2008) IL-6 receptor inhibition with tocilizumab improves treatment outcomes in patients with rheumatoid arthritis refractory to anti-tumour necrosis factor biologicals: results from a 24-week multicentre randomised placebo-controlled trial, *Ann Rheum Dis*, 67, pp. 1516–1523. doi:10.1136/ard.2008.092932.

do Espírito Santo, R.C., Pompermayer, M.G., Bini, R.R., Olszewski, V., Teixeira, E.G., Chakr, R., Xavier, R.M. and Brenol, C.V. (2018) Neuromuscular fatigue is weakly associated with perception of fatigue and function in patients with rheumatoid arthritis, *Rheumatology International*, 38(3), pp. 415–423. doi:10.1007/s00296-017-3894-z.

Stellenbosch University https://scholar.sun.ac.za

Evans, W.J. *et al.* (2008) Cachexia: A new definition, *Clinical Nutrition*, 27(6), pp. 793–799. doi:10.1016/j.clnu.2008.06.013.

Fang, C., Li, B., Wang, J.J., Fischer, J.E. and Hasselgren, P. (1998) Treatment of burned rats with insulin-like growth factor I inhibits the catabolic response in skeletal muscle, *American Physiological Society*, 275(4), pp. 1091–1098.

Farrow, M., Biglands, J., Tanner, S., Hensor, E.M.A., Buch, M.H., Emery, P. and Tan, A.L. (2021) Muscle deterioration due to rheumatoid arthritis: Assessment by quantitative MRI and strength testing, *Rheumatology (United Kingdom)*, 60(3), pp. 1216–1225. doi:10.1093/rheumatology/keaa364.

Filippin, L.I., Teixeira, V.N., Viacava, P.R., Lora, P.S., Xavier, L.L. and Xavier, R.M. (2013) Temporal development of muscle atrophy in murine model of arthritis is related to disease severity, *Journal of Cachexia, Sarcopenia and Muscle*, 4(3), pp. 231–238. doi:10.1007/s13539-013-0102-1.

Finol, H.J., Muller, B., Montes de Oca, I. and Marquez, A. (1988) Ultrastructure of skeletal muscle in rheumatoid myositis, *Journal of Rheumatology*, 15(4), pp. 552–555.

Firestein, G.S. (2003) Evolving concepts of rheumatoid arthritis, *Nature*, 423(6937), pp. 356–361. doi:10.1038/nature01661.

Flores, E.A., Bistrian, B.R., Pomposelli, J.J., Dinarello, C.A., Blackburn, G.L. and Istfan, N.W. (1989) Infusion of tumor necrosis factor/cachectin promotes muscle catabolism in the rat . A synergistic effect with interleukin 1, *The American Society for Clinical Investigation, Inc*, 83, pp. 1614–1622.

Frier, B.C., Noble, E.G. and Locke, M. (2008) Diabetes-induced atrophy is associated with a muscle-specific alteration in NF- κB activation and expression, *Cell Stress and Chaperones*, 13, pp. 287–296. doi:10.1007/s12192-008-0062-0.

Fry, C.S., Lee, J.D., Jackson, J.R., Kirby, T.J., Stasko, S.A., Liu, H., Dupont-Versteegden, E.E., McCarthy, J.J. and Peterson, C.A. (2014) Regulation of the muscle fiber microenvironment by activated satellite cells during hypertrophy, *The FASEB Journal*, 28, pp. 1654–1665. doi:10.1096/fj.13-239426.

Fry, C.S., Kirby, T.J., Kosmac, K., McCarthy, J.J. and Peterson, C.A. (2017)

Stellenbosch University https://scholar.sun.ac.za

Myogenic progenitor cells control extracellular matrix production by fibroblasts during skeletal muscle hypertrophy, *Stem Cell*, 20(1), pp. 56–69. doi:10.1016/j.stem.2016.09.010.

Gaffo, A., Saag, K.G. and Curtis, J.R. (2006) Treatment of rheumatoid arthritis, *Clinical Reviews*, 63, pp. 2451–2465. doi:10.2146/ajhp050514.

Garg, K., Corona, B.T. and Walters, T.J. (2015) Therapeutic strategies for preventing skeletal muscle fibrosis after injury., *Frontiers in Pharmacology*, 6(April), p. 87. doi:10.3389/fphar.2015.00087.

Gilson, H., Schakman, O., Kalista, S., Lause, P., Tsuchida, K. and Thissen, J.-P. (2009) Follistatin induces muscle hypertrophy through satellite cell proliferation and inhibition of both myostatin and activin, *American Journal of Physiology - Endocrinology & Metabolism*, 297, pp. 157–164. doi:10.1152/ajpendo.00193.2009.

Giuliani, G., Rosina, M. and Reggio, A. (2021) Signaling pathways regulating the fate of fibro/adipogenic progenitors (FAPs) in skeletal muscle regeneration and disease, *FEBS Journal*, pp. 1–34. doi:10.1111/febs.16080.

Goetsch, K.P., Snyman, C., Myburgh, K.H. and Niesler, C.U. (2015) Simultaneous isolation of enriched myoblasts and fibroblasts for migration analysis within a novel co-culture assay, *BioTechniques*, 58(1), pp. 25–32. doi:10.2144/000114246.

Gómez-SanMiguel, A.B., Gomez-Moreira, C., Nieto-Bona, M.P., Fernández-Galaz, C., Villanúa, M.Á., Martín, A.I. and López-Calderón, A. (2016) Formoterol decreases muscle wasting as well as inflammation in the rat model of rheumatoid arthritis, *American Journal of Physiology - Endocrinology and Metabolism*, 310(11), pp. E925–E937. doi:10.1152/ajpendo.00503.2015.

Gonzalez-Gonzalez, F.J., Chandel, N.S., Jain, M. and Budinger, G.R.S. (2017) Reactive oxygen species as signaling molecules in the development of lung fibrosis, *Translational Research*, 190, pp. 61–68. doi:10.1016/j.trsl.2017.09.005.

Grzelkowska-Kowalczyk, K. (2016) The Importance of Extracellular Matrix in Skeletal Muscle Development and Function, *Composition and Function of the Extracellular Matrix in the Human Body*, pp. 3–24. doi:10.5772/62230.

Gudagudi, K.B., d'Entrèves, N.P., Woudberg, N.J., Steyn, P.J. and Myburgh, K.H.

Stellenbosch University https://scholar.sun.ac.za

(2020) In vitro induction of quiescence in isolated primary human myoblasts, *Cytotechnology*, 72(2), pp. 189–202. doi:10.1007/s10616-019-00365-8.

Gumucio, J.P., Flood, M.D., Phan, A.C., Brooks, S. V and Mendias, C.L. (2013) Targeted inhibition of TGF-β results in an initial improvement but long-term deficit in force production after contraction-induced skeletal muscle injury., *Journal of Applied Physiology*, 115(4), pp. 539–545. doi:10.1152/japplphysiol.00374.2013.

Haddad, F., Zaldivar, F., Cooper, D.M. and Adams, G.R. (2005) IL-6-induced skeletal muscle atrophy, *Journal of Applied Physiology*, 98(3), pp. 911–917. doi:10.1152/japplphysiol.01026.2004.

von Haehling, S. and Anker, S.D. (2010) Cachexia as a major underestimated and unmet medical need: Facts and numbers, *Journal of Cachexia, Sarcopenia and Muscle*, 1(1), pp. 1–5. doi:10.1007/s13539-010-0002-6.

Hakkinen, A., Sokka, T., Kotaniemi, A. and Hannonen, P. (2001) A randomized two-year study of the effects of dynamic strength training on muscle strength, disease activity, functional capacity, and bone mineral density in early rheumatoid arthritis, *Arthritis and Rheumatism*, 44(3), pp. 515–522. doi:10.1002/1529-0131(200103)44:3<515::AID-ANR98>3.0.CO;2-5.

Häkkinen, A., Sokka, T., Kotaniemi, A., Paananen, M.L., Mälkiä, E., Kautiainen, H. and Hannonen, P. (1999) Muscle strength characteristics and central bone mineral density in women with recent onset rheumatoid arthritis compared with health controls, *Scandinavian Journal of Rheumatology*, 28(3), pp. 145–151. doi:10.1080/03009749950154202.

Hanaoka, B.Y., Peterson, C.A. and Crofford, L.J. (2012) Glucocorticoid effects on skeletal muscle: Benefit and risk in patients with autoimmune inflammatory rheumatoid diseases, *Expert Review of Clinical Immunology*, 8(8), pp. 695–697. doi:10.1586/eci.12.76.

Hartog, A., Hulsman, J. and Garssen, J. (2009) Locomotion and muscle mass measures in a murine model of collagen-induced arthritis, *BMC Musculoskeletal Disorders*, 10(1), pp. 1–7. doi:10.1186/1471-2474-10-59.

Hawkins, P., Armstrong, R., Boden, T., Garside, P., Knight, K., Lilley, E., Seed, M.,

Stellenbosch University https://scholar.sun.ac.za

Wilkinson, M. and Williams, R.O. (2015) Applying refinement to the use of mice and rats in rheumatoid arthritis research, *Inflammopharmacology*, 23(4), pp. 131–150. doi:10.1007/s10787-015-0241-4.

Helliwell, P.S. and Jackson, S. (1994) Relationship between weakness and muscle wasting in rheumatoid arthritis, *Annals of the Rheumatic Diseases*, 53(11), pp. 726–728. doi:10.1136/ard.53.11.726.

Henningsen, J., Rigbolt, K.T.G., Blagoev, B., Pedersen, B.K. and Kratchmarova, I. (2010) Dynamics of the skeletal muscle secretome during myoblast differentiation, *Molecular and Cellular Proteomics*, 9(11), pp. 2482–2496. doi:10.1074/mcp.M110.002113.

Heredia, J.E., Mukundan, L., Chen, F.M., Mueller, A.A., Deo, R.C., Locksley, R.M., Rando, T.A. and Chawla, A. (2013) Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration, *Cell*, 153, pp. 376–388. doi:10.1016/j.cell.2013.02.053.

Hogarth, M.W., Defour, A., Lazarski, C., Gallardo, E., Manera, J.D., Partridge, T.A., Nagaraju, K. and Jaiswal, J.K. (2019) Fibroadipogenic progenitors are responsible for muscle loss in limb girdle muscular dystrophy 2B, *Nature Communications*, 10(1), pp. 1–13. doi:10.1038/s41467-019-10438-z.

Horai, N. *et al.* (2013) Muscle wasting associated with pathologic change is a risk factor for the exacerbation of joint swelling in collagen-induced arthritis in cynomolgus monkeys, *BMC Musculoskeletal Disorders*, 14(1), p. 1. doi:10.1186/1471-2474-14-205.

Horsburgh, S., Robson-Ansley, P., Adams, R. and Smith, C. (2015) Exercise and inflammation-related epigenetic modifications: focus on DNA methylation, *Exercise, Inflammation, and DNA Methylation*, 2015(21), pp. 26–41.

Huffman, K.M. *et al.* (2017) Molecular alterations in skeletal muscle in rheumatoid arthritis are related to disease activity, physical inactivity, and disability, *Arthritis Research & Therapy*, 19(12), pp. 1–17.

Ismail, E., Nofal, O.K.J., Sakthiswary, R., Shaharir, S.S. and Sridharan, R. (2016) The clinical significance of interleukin-1 receptor antagonist +2018 polymorphism in

Stellenbosch University https://scholar.sun.ac.za

rheumatoid arthritis, *PLoS ONE*, 11(4), pp. 1–7. doi:10.1371/journal.pone.0153752.

Izumi, N. *et al.* (2006) BMP-7 opposes TGF-β1-mediated collagen induction in mouse pulmonary myofibroblasts through Id2, *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 290(1), pp. 1–4. doi:10.1152/ajplung.00171.2005.

Jen, Y., Weintraub, H. and Benezra, R. (1992) Overexpression of Id protein inhibits the muscle differentiation program: In vivo association of Id with E2A proteins, *Genes and Development*, 6(8), pp. 1466–1479. doi:10.1101/gad.6.8.1466.

Jin, J.-O., Han, X. and Yu, Q. (2013) Interleukin-6 induces the generation of IL-10-producing Tr1 cells and suppresses autoimmune tissue inflammation, *J Autoimmun*, 40(1), pp. 28–44. doi:10.1016/j.jaut.2012.07.009.Interleukin-6.

Joe, A.W.B., Yi, L., Natarajan, A., Le Grand, F., So, L., Wang, J., Rudnicki, M.A. and Rossi, F.M. V (2010) Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis, *Nat Cell Biol*, 12(2), pp. 153–163. doi:10.1038/ncb2015.Muscle.

Kang, X. *et al.* (2018) Interleukin-15 facilitates muscle regeneration through modulation of fibro/adipogenic progenitors, *Cell Communication and Signaling*, 16(42), pp. 1–11.

Kannan, K., Ortmann, R.A. and Kimpel, D. (2005) Animal models of rheumatoid arthritis and their relevance to human disease, *Pathophysiology*, 12(3), pp. 167–181. doi:10.1016/j.pathophys.2005.07.011.

Kay, J. and Calabrese, L. (2004) The role of interleukin-1 in the pathogenesis of rheumatoid arthritis, *Rheumatology*, 43(Suppl. 3), pp. iii2–iii9. doi:10.1007/s11926-009-0052-y.

Kesharwani, D., Paliwal, R., Satapathy, T. and Paul, S. Das (2019) Rheumatiod arthritis: An updated overview of latest therapy and drug delivery, *Journal of Pharmacopuncture*, 22(4), pp. 210–224.

Kharraz, Y., Guerra, J., Mann, C.J., Serrano, A.L. and Muñoz-cánoves, P. (2013) Macrophage Plasticity and the Role of Inflammation in Skeletal Muscle Repair, *Mediators of Inflammation*, 2013, pp. 1–9.

Stellenbosch University https://scholar.sun.ac.za

Khoja, S.S., Moore, C.G., Goodpaster, B.H., Delitto, A. and Piva, S.R. (2018) Skeletal muscle fat and its association with physical function in Rheumatoid Arthritis, *Arthritis Care & Research*, 70(3), pp. 333–342. doi:10.1002/acr.

Kim, J. and Lee, J. (2017) Role of transforming growth factor-β in muscle damage and regeneration: Focused on eccentric muscle contraction, *Journal of Exercise Rehabilitation*, 13(6), pp. 621–626. doi:10.12965/jer.1735072.536.

Kim, R.C. and Collins, G.H. (1981) The neuropathology of rheumatoid disease, *Human Pathology*, 12(1), pp. 5–15. doi:10.1016/S0046-8177(81)80236-5.

Kinne, R.W., Brauer, R., Stuhlmuller, B., Palombo-Kinne, E. and Burmester, G.-R. (2000) Macrophages in rheumatoid arthritis, *Arthritis Research*, 2(3), pp. 189–202.

Kohn, T.A. and Myburgh, K.H. (2007) Regional specialization of rat quadriceps myosin heavy chain isoforms occurring in distal to proximal parts of middle and deep regions is not mirrored by citrate synthase activity, *Journal of Anatomy*, 210(1), pp. 8–18. doi:10.1111/j.1469-7580.2007.00661.x.

Koufany, M., Jouzeau, J. and Moulin, D. (2014) Fenofibrate vs pioglitazone: Comparative study of the anti-arthritic potencies of PPAR-alpha and PPAR-gamma agonists in rat adjuvant-induced arthritis, *Bio-Medical Materials and Engineering*, 24, pp. 81–89. doi:10.3233/BME-140977.

Kozakowska, M. and Alicja, K.P. (2015) The role of oxidative stress in skeletal muscle injury and regeneration : focus on antioxidant enzymes, *Journal of Muscle Research and Cell Motility* [Preprint]. doi:10.1007/s10974-015-9438-9.

Kramer, H.R., Fontaine, K.R., Bathon, J.M. and Giles, J.T. (2012) Muscle density in rheumatoid arthritis: Associations with disease features and functional outcomes, *Arthritis and Rheumatism*, 64(8), pp. 2438–2450. doi:10.1002/art.34464.

Kühl, U., Timpl, R. and von der Mark, K. (1982) Synthesis of type IV collagen and laminin in cultures of skeletal muscle cells and their assembly on the surface of myotubes, *Developmental Biology*, 93(2), pp. 344–354. doi:10.1016/0012-1606(82)90122-1.

Kung, C.C., Dai, S.P., Chiang, H., Huang, H.S. and Sun, W.H. (2020) Temporal expression patterns of distinct cytokines and M1/M2 macrophage polarization

Stellenbosch University https://scholar.sun.ac.za

regulate rheumatoid arthritis progression, *Molecular Biology Reports*, 47(5), pp. 3423–3437. doi:10.1007/s11033-020-05422-6.

Lazúrová, I. and Tomás, Ľ. (2017) Cardiac impairment in rheumatoid arthritis and influence of anti-TNFα treatment, *Clinical Reviews in Allergy & Immunology*, 52, pp. 323–332. doi:10.1007/s12016-016-8566-3.

Lemmey, A.B. *et al.* (2016) Tight control of disease activity fails to improve body composition or physical function in rheumatoid arthritis patients, *Rheumatology (United Kingdom)*, 55(10), pp. 1736–1745. doi:10.1093/rheumatology/kew243.

Lemos, D.R. *et al.* (2015) Nilotinib reduces muscle fibrosis in chronic muscle injury by promoting TNF-mediated apoptosis of fibro/adipogenic progenitors, *Nature Medicine*, 21(7), pp. 786–794. doi:10.1038/nm.3869.

Lertnawapan, R., Chonprasertsuk, S. and Siramolpiwat, S. (2018) Association between cumulative methotrexate dose,- non-invasive scoring system and hepatic fibrosis detected by Fibroscan in rheumatoid arthritis patients receiving methotrexate, *International Journal of Rheumatic Diseases*, 22(2), pp. 214–221. doi:10.1111/1756-185X.13442.

Li, H., Malhotra, S. and Kumar, A. (2008) Nuclear factor-kappa B signaling in skeletal muscle atrophy, *Journal of Molecular Medicine*, 86, pp. 1113–1126. doi:10.1007/s00109-008-0373-8.

Li, P., Waters, R.E., Redfern, S.I., Zhang, M., Mao, L., Annex, B.H. and Yan, Z. (2007) Oxidative phenotype protects myofibers from pathological insults induced by chronic heart failure in mice, *Musculoskeletal Pathology*, 170(2), pp. 599–608. doi:10.2353/ajpath.2007.060505.

Li, Y., Chen, Y., John, J., Moylan, J., Jin, B., Mann, D.L. and Reid, M.B. (2005) TNF-a acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin/MAFbx in skeletal muscle, *FASEB Journal*, 19(3), pp. 362–370. doi:10.1096/fj.04-2364com.TNF-.

Lin, G.G. and Scott, J.G. (2007) Skeletal muscle satellite cells and adult myogenesis, *Current Opinion in Cell Biology*, 19(6), pp. 628–633. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2215059/pdf/nihms36360.pdf.

Stellenbosch University https://scholar.sun.ac.za

Lindberg, J. *et al.* (2010) The gene expression profile in the synovium as a predictor of the clinical response to infliximab treatment in rheumatoid arthritis, *PLoS ONE*, 5(6), pp. 1–7. doi:10.1371/journal.pone.0011310.

Lindström, M. (2009) Satellite cells in human skeletal muscle: molecular identification, quantification and function, *Umea University Medical Dissertations*, 1313, pp. 1–56.

Lipsky, P.E. *et al.* (2000) Infliximab and Methotrexate in the treatment of rheumatoid arthritis, *The New England Journal of Medicine*, 343(22), pp. 1594–1602.

Little, R.D., Prieto-Potin, I., Pérez-Baos, S., Villalvilla, A., Gratal, P., Cicuttini, F., Largo, R. and Herrero-Beaumont, G. (2017) Compensatory anabolic signaling in the sarcopenia of experimental chronic arthritis, *Scientific Reports*, 7(1), pp. 1–11. doi:10.1038/s41598-017-06581-6.

Liu, C., Ding, B., Wang, H. and Lengyel, P. (2002) The MyoD-Inducible p204 Protein Overcomes the Inhibition of Myoblast Differentiation by Id Proteins, *Molecular and Cellular Biology*, 22(9), pp. 2893–2905. doi:10.1128/mcb.22.9.2893-2905.2002.

Liu, R.M. and Desai, L.P. (2015) Reciprocal regulation of TGF-β and reactive oxygen species: A perverse cycle for fibrosis, *Redox Biology*, 6, pp. 565–577. doi:10.1016/j.redox.2015.09.009.

Londhe, P. and Guttridge, D.C. (2015) Inflammation induced loss of skeletal muscle, *Bone*, 80, pp. 131–142. doi:10.1016/S2215-0366(16)30284-X.Epidemiology.

López-Menduiña, M., Martín, A.I., Castillero, E., Villanúa, M.A. and López-Calderón, A. (2010) Systemic IGF-I administration attenuates the inhibitory effect of chronic arthritis on gastrocnemius mass and decreases atrogin-1 and IGFBP-3, *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 299(2), pp. R541–R551. doi:10.1152/ajpregu.00211.2010.

Maezawa, T., Tanaka, M., Kanazashi, M., Maeshige, N., Kondo, H., Ishihara, A. and Fujino, H. (2017) Astaxanthin supplementation attenuates immobilization-induced skeletal muscle fibrosis via suppression of oxidative stress, *Journal of Physiological Sciences*, 67(5), pp. 603–611. doi:10.1007/s12576-016-0492-x.

Magyar E, Talerman A, Mohácsy J, Wouters HW, de B.W. (1977) Muscle changes

Stellenbosch University https://scholar.sun.ac.za

in rheumatoid arthritis, *Virchows Archiv A Pathological Anatomy and Histology*, 373(3), pp. 267–278. doi:10.1007/BF00432241.

Mahdy, M.A.A. (2019) Skeletal muscle fibrosis: an overview, *Cell and Tissue Research*, 375, pp. 575–588.

Majithia, V. and Geraci, S.A. (2007) Rheumatoid Arthritis: diagnosis and management, *The American Journal of Medicine*, 120, pp. 936–939. doi:10.1016/j.amjmed.2007.04.005.

Maksimovic, V., Pavlovic-Popovic, Z., Vukmirovic, S., Cvejic, J., Mooranian, A., Al-Salami, H., Mikov, M. and Golocorbin-Kon, S. (2020) Molecular mechanism of action and pharmacokinetic properties of methotrexate, *Molecular Biology Reports* [Preprint], (0123456789). doi:10.1007/s11033-020-05481-9.

Mann, C.J., Perdiguero, E., Kharraz, Y., Aguilar, S., Pessina, P., Serrano, A.L. and Muñoz-Cánoves, P. (2011) Aberrant repair and fibrosis development in skeletal muscle, *Skeletal Muscle*, 1(1), p. 21. doi:10.1186/2044-5040-1-21.

Marinello, P.C. *et al.* (2018) Isoflavin-beta modifies muscle oxidative stress and prevents a thyrotoxicosis-induced loss of muscle mass in rats, *Muscle Nerve*, 56(5), pp. 975–981.

Masuko, K. (2014) Rheumatoid cachexia revisited: A metabolic co-morbidity in rheumatoid arthritis, *Frontiers in Nutrition*, 1(November), pp. 1–7. doi:10.3389/fnut.2014.00020.

Matschke, V., Murphy, P., Lemmey, A.B., Maddison, P.J. and Thom, J.M. (2010) Muscle quality, architecture, and activation in cachectic patients with rheumatoid arthritis, *Journal of Rheumatology*, 37(2), pp. 282–284. doi:10.3899/jrheum.090584.

Matschke, V., Murphy, P., Lemmey, A.B., Maddison, P. and Thom, J.M. (2010) Skeletal muscle properties in Rheumatoid arthritis patients, *Medicine and Science in Sports and Exercise*, 42(12), pp. 2149–2155. doi:10.1249/MSS.0b013e3181e304c3.

Mcinnes, I.B. and Schett, G. (2011) The pathogenesis of rheumatoid arthritis., *The New England Journal of Medicine*, 365(23), pp. 2206–2219.

McInnes, I.B. and Schett, G. (2017) Targeted treatments for rheumatoid arthritis 1: Pathogenetic insights from the treatment of rheumatoid arthritis, *The Lancet*, 389,

Stellenbosch University https://scholar.sun.ac.za

pp. 2328–2337. doi:10.1016/S0140-6736(17)31472-1.

McVicker, B.L. and Bennett, R.G. (2017) Novel anti-fibrotic therapies, *Frontiers in Pharmacology*, 8(MAY), pp. 1–21. doi:10.3389/fphar.2017.00318.

Menck, K., Behme, D., Pantke, M., Reiling, N., Binder, C., Pukrop, T. and Klemm, F. (2014) Isolation of human monocytes by double gradient centrifugation and their differentiation to macrophages in Teflon-coated cell culture bags, *Journal of Visualized Experiments*, (91), pp. 1–10. doi:10.3791/51554.

Michaëlssony, E., Holmdahl, M., Engström, Å., Burkhardt, H., Scheynius, A. and Holmdahl, R. (1995) Macrophages, but not dendritic cells, present collagen to T cells, *European Journal of Immunology*, 25(8), pp. 2234–2241. doi:10.1002/eji.1830250818.

Miki, Y., Ono, K., Hata, S., Suzuki, T., Kumamoto, H. and Sasano, H. (2012) The advantages of co-culture over mono cell culture in simulating in vivo environment, *Journal of Steroid Biochemistry and Molecular Biology*, 131(3–5), pp. 68–75. doi:10.1016/j.jsbmb.2011.12.004.

Minnaard, R., Drost, M.R., Wagenmakers, A.J.M., van Kranenburg, G.P., Kuipers, H. and Hesselink, M.K.C. (2005) Skeletal muscle wasting and contractile performance in septic rats, *Muscle & Nerve*, 31, pp. 339–348. doi:10.1002/mus.20268.

Misharin, A. V *et al.* (2014) Non-classical Ly6C- monocytes drive the development of inflammatory arthritis in mice, *Cell Rep*, (2), pp. 591–604. doi:10.1016/j.celrep.2014.09.032.Non-classical.

Mizwicki, M.T. *et al.* (2012) Tocilizumab attenuates inflammation in ALS patients through inhibition of IL6 receptor signaling, *American Journal of Neurodegenerative Diseases*, 1(3), pp. 305–315.

Muñoz-Cánoves, P., Scheele, C., Pedersen, B.K. and Serrano, A.L. (2013) Interleukin-6 myokine signaling in skeletal muscle: A double-edged sword?, *FEBS Journal*, 280(17), pp. 4131–4148. doi:10.1111/febs.12338.

Narváez, J. (2016) Treatment of rheumatoid arthritis, *Medicina Clínica (English Edition)*, 147(4), pp. 176–180. doi:10.1016/j.medcle.2016.09.023.

Nederveen, J.P., Joanisse, S., Snijders, T., Ivankovic, V., Baker, S.K., Phillips, S.M.

Stellenbosch University https://scholar.sun.ac.za

and Parise, G. (2016) Skeletal muscle satellite cells are located at a closer proximity to capillaries in healthy young compared with older men, *Journal of Cachexia, Sarcopenia and Muscle*, 7, pp. 547–554. doi:10.1002/jcsm.12105.

de Oliveira Nunes Teixeira, V., Filippin, L.I., Viacava, P.R., de Oliveira, P.G. and Xavier, R.M. (2013) Muscle wasting in collagen-induced arthritis and disuse atrophy, *Experimental Biology and Medicine*, 238(12), pp. 1421–1430. doi:10.1177/1535370213505961.

Ollewagen, T., Myburgh, K.H., Vyver, M. Van De and Smith, C. (2021) Rheumatoid cachexia: the underappreciated role of myoblast, macrophage and fibroblast interplay in the skeletal muscle niche, *Journal of Biomedical Science*, 28(15), pp. 1–16. doi:10.1186/s12929-021-00714-w.

Ollewagen, T., Powrie, Y.S.L., Myburgh, K.H. and Smith, C. (2021) Unresolved intramuscular inflammation, not diminished skeletal muscle regenerative capacity, is at the root of rheumatoid cachexia: insights from a rat CIA model, *Physiological Reports*, 9(22), pp. 1–15. doi:10.14814/phy2.15119.

Ott, L.W. *et al.* (2007) Tumor necrosis factor-α -and interleukin-1-induced cellular responses: coupling proteomic and genomic information, *J Proteome Res*, 6(6), pp. 2176–2185. doi:10.1021/pr060665l.Tumor.

Oyenihi, A.B., Ollewagen, T., Myburgh, K.H., Powrie, Y.S.L. and Smith, C. (2019) Redox status and muscle pathology in rheumatoid arthritis: Insights from various rat hindlimb muscles, *Oxidative Medicine and Cellular Longevity*, 2019, pp. 1–11.

Pedersen, B.K. and Febbraio, M.A. (2012) Muscles, exercise and obesity: Skeletal muscle as a secretory organ, *Nature Reviews Endocrinology*, 8(8), pp. 457–465. doi:10.1038/nrendo.2012.49.

Pedersen, B.K., Steensberg, A. and Schjerling, P. (2001) Muscle-derived interleukin-6: Possible biological effects, *Journal of Physiology*, 536(2), pp. 329–337. doi:10.1111/j.1469-7793.2001.0329c.xd.

Perandini, L.A., Chimin, P., Lutkemeyer, D. da S. and Câmara, N.O.S. (2018) Chronic inflammation in skeletal muscle impairs satellite cells function during regeneration: can physical exercise restore the satellite cell niche?, *FEBS Journal*,

Stellenbosch University https://scholar.sun.ac.za

285(11), pp. 1973–1984. doi:10.1111/febs.14417.

Perricone, C. *et al.* (2019) Porphyromonas gingivalis and rheumatoid arthritis, *Current Opinion in Rheumatology*, 31(5), pp. 517–524. doi:10.1097/bor.0000000000000649.

Perry, B.D. *et al.* (2016) Muscle atrophy in patients with Type 2 Diabetes Mellitus: roles of inflammatory pathways, physical activity and exercise, *Exercise Immunology Reviews*, 22, pp. 94–109.

Pinho, R.A., Sepa-Kishi, D.M., Bikopoulos, G., Wu, M. V., Uthayakumar, A., Mohasses, A., Hughes, M.C., Perry, C.G.R. and Ceddia, R.B. (2017) High-fat diet induces skeletal muscle oxidative stress in a fiber type-dependent manner in rats, *Free Radical Biology and Medicine*, 110(July), pp. 381–389. doi:10.1016/j.freeradbiomed.2017.07.005.

Pironti, G., Bersellini-Farinotti, A., Agalave, N.M., Sandor, K., Fernandez-Zafra, T., Jurczak, A., Lund, L.H., Svensson, C.I. and Andersson, D.C. (2018) Cardiomyopathy, oxidative stress and impaired contractility in a rheumatoid arthritis mouse model, *Heart*, 0, pp. 1–9. doi:10.1136/heartjnl-2018-312979.

Prestigiacomo, V., Weston, A., Messner, S., Lampart, F. and Suter-Dick, L. (2017) Pro-fibrotic compounds induce stellate cell activation, ECM-remodelling and Nrf2 activation in a human 3D-multicellular model of liver fibrosis, *PLoS ONE*, 12(6), pp. 1–17.

Quéméneur, L., Gerland, L., Flacher, M., Ffrench, M., Revillard, J.-P. and Genestier, L. (2003) Differential control of cell cycle, proliferation, and survival of primary T lymphocytes by purine and pyrimidine nucleotides, *Journal of Immunology*, 170, pp. 4986–4995. doi:10.4049/jimmunol.170.10.4986.

Rajaiah, R. and Moudgil, K. (2009) Animal models, in *Rheumatoid Arthritis*, pp. 218–224. doi:10.1016/0738-081X(83)90026-3.

Rebbapragada, A., Benchabane, H., Wrana, J.L., Celeste, A.J. and Attisano, L. (2003) Myostatin Signals through a Transforming Growth Factor β-Like Signaling Pathway To Block Adipogenesis, *Molecular and Cellular Biology*, 23(20), pp. 7230–7242. doi:10.1128/mcb.23.20.7230-7242.2003.

Stellenbosch University https://scholar.sun.ac.za

Rech, J., Hess, A., Finzel, S., Kreitz, S., Sergeeva, M., Englbrecht, M., Doerfler, A., Saake, M. and Schett, G. (2013) Association of brain functional magnetic resonance activity with response to tumor necrosis factor inhibition in rheumatoid arthritis, *Arthritis and Rheumatism*, 65(2), pp. 325–333. doi:10.1002/art.37761.

Reid, M.B. and Moylan, J.S. (2011) Beyond atrophy: Redox mechanisms of muscle dysfunction in chronic inflammatory disease, *Journal of Physiology*, 589(9), pp. 2171–2179. doi:10.1113/jphysiol.2010.203356.

Remels, A.H.V., Gosker, H.R., Langen, R.C.J. and Schols, A.M.W.J. (2013) The mechanisms of cachexia underlying muscle dysfunction in COPD, *Journal of Applied Physiology*, 114(9), pp. 1253–1262. doi:10.1152/japplphysiol.00790.2012.

Rigamonti, E., Zordan, P., Sciorati, C., Rovere-Querini, P. and Brunelli, S. (2014) Macrophage plasticity in skeletal muscle repair, *BioMed Research International*, 2014. doi:10.1155/2014/560629.

Ross, K., Powrie, Y. and Smith, C. (2020) Accelerated ageing profile in rheumatoid arthritis is unique and tissue compartment specific, *Inflammopharmacology*, In press. doi:10.1007/s10787-020-00731-5.

Roubenoff, R., Roubenoff, R.A., Cannon, J.G., Kehayias, J.J., Zhuang, H., Dawson-hughes, B., Dinarello, C.A. and Rosenberg, I.H. (1994) Rheumatoid cachexia: cytokine-driven hypermetabolism accompanying reduced body cell mass in chronic inflammation, *The Journal of Clinical Investigation*, 93, pp. 2379–2386.

Roubenoff, R., Freeman, L.M., Smith, D.E., Abad, L.W., Dinarello, C.A. and Kehayias, J.J. (1997) Adjuvant arthritis as a model of inflammatory cachexia, *Arthritis and Rheumatism*, 40(3), pp. 534–539. doi:10.1002/art.1780400320.

Santiago, J.J. *et al.* (2010) Cardiac fibroblast to myofibroblast differentiation in vivo and in vitro: Expression of focal adhesion components in neonatal and adult rat ventricular myofibroblasts, *Developmental Dynamics*, 239(6), pp. 1573–1584. doi:10.1002/dvdy.22280.

Santo, Rafaela C E, Fernandes, K.Z., Lora, P.S. and Filippin, L.I. (2018) Prevalence of rheumatoid cachexia in rheumatoid arthritis : a systematic review and meta-analysis, *Journal of Cachexia, Sarcopenia and Muscle*, 9(5), pp. 816–825.

Stellenbosch University https://scholar.sun.ac.za

doi:10.1002/jcsm.12320.

Santo, Rafaela C.E., Fernandes, K.Z., Lora, P.S., Filippin, L.I. and Xavier, R.M. (2018) Prevalence of rheumatoid cachexia in rheumatoid arthritis: a systematic review and meta-analysis, *Journal of Cachexia, Sarcopenia and Muscle*, 9(5), pp. 816–825. doi:10.1002/jcsm.12320.

Schabort, E.J., van der Merwe, M., Loos, B., Moore, F.P. and Niesler, C.U. (2009) TGF-β's delay skeletal muscle progenitor cell differentiation in an isoform-independent manner, *Experimental Cell Research*, 315, pp. 373–384. doi:10.1016/j.yexcr.2008.10.037.

Schulert, G.S., Fall, N., Harley, J.B., Shen, N., Lovell, D.J., Thornton, S. and Grom, A.A. (2016) Monocyte microRNA expression in active systemic juvenile idiopathic arthritis implicates miR-125a-5p in polarized monocyte phenotypes, *Arthritis & Rheumatology*, 68(9), pp. 2300–2313. doi:10.1002/art.39694.Monocyte.

Schuster, R., Motola-Kalay, N., Baranovski, B.M., Bar, L., Tov, N., Stein, M., Lewis, E.C., Ayalon, M. and Sagiv, Y. (2020) Distinct anti-inflammatory properties of alpha1-antitrypsin and corticosteroids reveal unique underlying mechanisms of action, *Cellular Immunology*, 356(July), p. 104177. doi:10.1016/j.cellimm.2020.104177.

Seidel, M., Rozwadowska, N., Tomczak, K. and Kurpisz, M. (2006) Myoblast preparation for transplantation into injured myocardium, *European Heart Journal, Supplement*, 8(H), pp. 8–15. doi:10.1093/eurheartj/sul061.

Serrano, A.L., Baeza-Raja, B., Perdiguero, E., Jardí, M. and Muñoz-Cánoves, P. (2008) Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy, *Cell Metabolism*, 7(1), pp. 33–44. doi:10.1016/j.cmet.2007.11.011.

Serrano, A.L., Mann, C.J., Vidal, B., Ardite, E., Perdiguero, E. and Munoz-Canoves, P. (2011) Cellular and molecular mechanisms regulating fibrosis in skeletal muscle repair and disease, *Current Topics in Developmental Biology*, 96, pp. 167–201. doi:10.1016/B978-0-12-385940-2.00007-3.

Serrano, A.L. and Muñoz-cánoves, P. (2010) Regulation and dysregulation of fibrosis in skeletal muscle, *Experimental Cell Research*, 316(18), pp. 3050–3058.

Stellenbosch University https://scholar.sun.ac.za

doi:10.1016/j.yexcr.2010.05.035.

Servais, S., Letexier, D., Favier, R., Duchamp, C. and Desplanches, D. (2007) Prevention of unloading-induced atrophy by vitamin E supplementation: Links between oxidative stress and soleus muscle proteolysis?, *Free Radical Biology and Medicine*, 42(5), pp. 627–635. doi:10.1016/j.freeradbiomed.2006.12.001.

Shiratori, H., Feinweber, C., Luckhardt, S., Wallner, N., Geisslinger, G., Weigert, A. and Parnham, M.J. (2018) An in vitro test system for compounds that modulate human inflammatory macrophage polarization, *European Journal of Pharmacology*, 833, pp. 328–338. doi:10.1016/j.ejphar.2018.06.017.

Singla, D.K., Rocher, C., Singla, R., Singal, P.K. and Parthasarathy, S. (2012) Bone morphogenetic protein 7 polarizes THP-1 cells into M2 macrophages, *Canadian Journal of Physiology and Pharmacology*, 90(7), pp. 947–951. doi:10.1139/Y2012-102.

Singla, D.K., Singla, R. and Wang, J. (2016) BMP-7 treatment increases M2 macrophage differentiation and reduces inflammation and plaque formation in Apo E-/-Mice, *PLoS ONE*, 11(1), pp. 1–17. doi:10.1371/journal.pone.0147897.

Sironi, M. *et al.* (2006) Differential regulation of chemokine production by Fcy receptor engagement in human monocytes: association of CCL1 with a distinct form of M2 monocyte activation (M2b, Type 2), *Journal of Leukocyte Biology*, 80, pp. 342–349. doi:10.1189/jlb.1005586.

Smith, C., Kruger, M.J., Smith, R.M. and Myburgh, K.H. (2008) The inflammatory response to skeletal muscle injury: Illuminating complexities, *Sports Medicine*, 38(11), pp. 947–969. doi:10.2165/00007256-200838110-00005.

Smith, C. and Swart, A.C. (2016) Rooibos (Aspalathus linearis) facilitates an anti-inflammatory state, modulating IL-6 and IL-10 while not inhibiting the acute glucocorticoid response to a mild novel stressor in vivo, *Journal of Functional Foods*, 27, pp. 42–54. doi:10.1016/j.jff.2016.08.055.

Smolen, J.S., Aletaha, D., Koeller, M., Weisman, M.H. and Emery, P. (2007) New therapies for treatment of rheumatoid arthritis, *Lancet*, 370, pp. 1861–1874. doi:10.1016/S0140-6736(07)60784-3.

Stellenbosch University https://scholar.sun.ac.za

Smolen, J.S. *et al.* (2020) EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological modifying antirheumatic drugs : 2019 update, *Ann Rheum Dis*, 0, pp. 1–15. doi:10.1136/annrheumdis-2019-216655.

Sokka, T. *et al.* (2008) Physical inactivity in patients with rheumatoid arthritis: Data from twenty-one countries in a cross-sectional, international study, *Arthritis Care and Research*, 59(1), pp. 42–50. doi:10.1002/art.23255.

Song, H.P., Li, X., Yu, R., Zeng, G., Yuan, Z.Y., Wang, W., Huang, H.Y. and Cai, X. (2015) Phenotypic characterization of type II collagen-induced arthritis in Wistar rats, *Experimental and Therapeutic Medicine*, 10(4), pp. 1483–1488. doi:10.3892/etm.2015.2667.

Steenvoorden, M.M.C. *et al.* (2006) Transition of healthy to diseased synovial tissue in rheumatoid arthritis is associated with gain of mesenchymal/fibrotic characteristics, *Arthritis Research and Therapy*, 8(6), pp. 2–11. doi:10.1186/ar2073.

Steinz, M.M. *et al.* (2019) Oxidative hotspots on actin promote skeletal muscle weakness in rheumatoid arthritis, *JCI Insight*, 4(9), pp. 1–16. doi:10.1172/jci.insight.126347.

Steyn, P.J., Dzobo, K., Smith, R.I. and Myburgh, K.H. (2019) Interleukin-6 induces myogenic differentiation via JAK2-STAT3 signaling in mouse C2C12 myoblast cell line and primary human myoblasts, *International Journal of Molecular Sciences*, 20(5273), pp. 1–30.

Summers, G.D., Deighton, C.M., Rennie, M.J. and Booth, A.H. (2008) Rheumatoid cachexia: A clinical perspective, *Rheumatology*, 47(8), pp. 1124–1131. doi:10.1093/rheumatology/ken146.

Sung, J., Hong, J., Kang, H., Choi, I., Lim, S.-D., Lee, J.-K., Seok, J.-H., Lee, J.-H. and Hur, G.-M. (2000) Methotrexate suppresses the interleukin-6 induced generation of reactive oxygen species in the synoviocytes of rheumatoid arthritis, *Immunopharmacology*, 47, pp. 35–44.

Takahashi, T., Muneta, T., Tsuji, K. and Sekiya, I. (2011) BMP-7 inhibits cartilage degeneration through suppression of inflammation in rat zymosan-induced arthritis, *Cell and Tissue Research*, 344(2), pp. 321–332. doi:10.1007/s00441-011-1154-1.

Stellenbosch University https://scholar.sun.ac.za

Tang, L., Zhang, H., Wang, C., Li, H., Zhang, Q. and Bai, J. (2017) M2A and M2C macrophage subsets amerliorate inflammation and fibroproliferation in acute lung injury through interleukin 10 pathway, *Shock*, 48(1), pp. 119–129. doi:10.1097/SHK.0000000000000820.

Tedeschi, S.K. and Costenbader, K.H. (2016) Is there a role for diet in the therapy of rheumatoid arthritis?, *Current Rheumatology Reports*, 18(23), pp. 1–9. doi:10.1007/s11926-016-0575-y.

Teixeira, V. de O.N., Filippin, L.I. and Xavier, R.M. (2012) Mechanisms of muscle wasting in sarcopenia, *Revista Brasileira de Reumatologia*, 52(2), pp. 247–253. doi:10.1590/S0482-50042012000200009.

Tidball, J.G., Welc, S.S. and Wehling-Henricks, M. (2018) Immunobiology of inherited muscular dystrophies, *Comprehensive Physiology*, 8, pp. 1313–1356. doi:10.1002/cphy.c170052.

Tournadre, A. *et al.* (2017) Changes in body composition and metabolic profile during interleukin 6 inhibition in rheumatoid arthritis, *Journal of Cachexia, Sarcopenia and Muscle*, 8(4), pp. 639–646. doi:10.1002/jcsm.12189.

Tsuneyoshi, Y., Tanaka, M., Nagai, T., Sunahara, N., Matsuda, T., Sonoda, T., Ijiri, K., Komiya, S. and Matsuyama, T. (2012) Functional folate receptor beta-expressing macrophages in osteoarthritis synovium and their M1/M2 expression profiles, *Scandinavian Journal of Rheumatology*, 41, pp. 132–140. doi:10.3109/03009742.2011.605391.

Udalova, I.A., Mantovani, A. and Feldmann, M. (2016) Macrophage heterogeneity in the context of rheumatoid arthritis, *Nature Publishing Group*, 12(8), pp. 472–485. doi:10.1038/nrrheum.2016.91.

Uutela, T.I., Kautiainen, H.J. and Häkkinen, A.H. (2018) Decreasing muscle performance associated with increasing disease activity in patients with rheumatoid arthritis, *PLoS ONE*, 13(4). doi:10.1371/journal.pone.0194917.

Vandooren, B. *et al.* (2009) Absence of a classically activated macrophage cytokine signature in peripheral spondylarthritis, including psoriatic arthritis, *Arthritis & Rheumatism*, 60(4), pp. 966–975. doi:10.1002/art.24406.

Stellenbosch University https://scholar.sun.ac.za

Vasanthi, P., Nalini, G. and Rajasekhar, G. (2007) Role of tumor necrosis factor-alpha in rheumatoid arthritis: A review, *APLAR Journal of Rheumatology*, 10(4), pp. 270–274. doi:10.1111/j.1479-8077.2007.00305.x.

Vidal, B. *et al.* (2008) Fibrinogen drives dystrophic muscle fibrosis via a TGF β/alternative macrophage activation pathway, *Genes & Development*, 22, pp. 1747–1752. doi:10.1101/gad.465908.

Visser, J.G. and Smith, C. (2018) Development of a transendothelial shuttle by macrophage modification, *Journal of Tissue Engineering and Regenerative Medicine*, 12(4), pp. 1889–1898. doi:10.1002/term.2620.

Visser, J.G., Van Staden, A.D.P. and Smith, C. (2019) Harnessing macrophages for controlled-release drug delivery: Lessons from microbes, *Frontiers in Pharmacology*, 10(22), pp. 1–18. doi:10.3389/fphar.2019.00022.

Walsmith, J. and Roubenoff, R. (2002) Cachexia in rheumatoid arthritis, *International Journal of Cardiology*, 85(1), pp. 89–99. doi:10.1016/S0167-5273(02)00237-1.

Wang, H., Melton, D.W., Porter, L., Sarwar, Z.U., McManus, L.M. and Shireman, P.K. (2014) Altered macrophage phenotype transition impairs skeletal muscle regeneration, *American Journal of Pathology*, 184(4), pp. 1167–1184. doi:10.1016/j.ajpath.2013.12.020.

Wang, L., Zhang, S., Wu, H., Rong, X. and Guo, J. (2019) M2b macrophage polarization and its roles in diseases, *Journal of Leukocyte Biology*, 106, pp. 345–358. doi:10.1002/JLB.3RU1018-378RR.

Wang, S., Wang, Shuang, Li, H., Zhu, L. and Wang, Y. (2019) Inhibition of the TGF-β/Smads signaling pathway attenuates pulmonary fibrosis and induces anti-proliferative effect on synovial fibroblasts in rheumatoid arthritis, *International Journal of Clinical Experimental Pathology*, 12(5), pp. 1835–1845.

Weiskirchen, R. and Meurer, S.K. (2013) BMP-7 counteracting TGF-beta 1 activities in organ fibrosis, *Frontiers in Bioscience*, 18, pp. 1407–1434.

Williams, R.O., Feldmann, M. and Maini, R.N. (1992) Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis, *Proceedings of the National Academy of Sciences of the United States of America*, 89(20), pp. 9784–

Stellenbosch University https://scholar.sun.ac.za

9788. doi:10.1073/pnas.89.20.9784.

Winbanks, C.E. *et al.* (2013) The bone morphogenetic protein axis is a positive regulator of skeletal muscle mass, *Journal of Cell Biology*, 203(2), pp. 345–357. doi:10.1083/jcb.201211134.

Wright, H.L., Bucknall, R.C., Moots, R.J. and Edwards, S.W. (2012) Analysis of SF and plasma cytokines provides insights into the mechanisms of inflammatory arthritis and may predict response to therapy, *Rheumatology*, 51(3), pp. 451–459. doi:10.1093/rheumatology/ker338.

Wroblewski, R., Gremski, W., Nordemar, R. and Edstrom, L. (1978) Electron probe x-ray microanalysis of human skeletal muscle involved in rheumatoid arthritis, *Histochemistry*, 57, pp. 1–8.

Wu, D.H. and Hatzopoulos, A.K. (2019) Bone morphogenetic protein signaling in inflammation, *Experimental Biology and Medicine*, 244(2), pp. 147–156. doi:10.1177/1535370219828694.

Wüst, R.C.I. and Degens, H. (2007) Factors contributing to muscle wasting and dysfunction in COPD patients, *International Journal of COPD*, 2(3), pp. 289–300.

Wynn, T.A. (2008) Cellular and molecular mechanisms of fibrosis, *Journal of Pathology*, 214, pp. 199–210. doi:10.1002/path.

Wynn, T.A. (2019) Two types of fibroblast drive arthritis, *Nature*, 570(7760), pp. 169–170. doi:10.1038/d41586-019-01594-9.

Xiao, W., Liu, Y. and Chen, P. (2016) Macrophage depletion impairs skeletal muscle regeneration: the roles of pro-fibrotic factors, inflammation, and oxidative stress, *Inflammation*, 39(6), pp. 2016–2028. doi:10.1007/s10753-016-0438-8.

Yamada, T. *et al.* (2009) Impaired myofibrillar function in the soleus muscle of mice with collagen-induced arthritis, *Arthritis and Rheumatism*, 60(11), pp. 3280–3289. doi:10.1002/art.24907.

Yamada, T. *et al.* (2015) Nitrosative modifications of the Ca2+release complex and actin underlie arthritis-induced muscle weakness, *Annals of the Rheumatic Diseases*, 74(10), pp. 1907–1914. doi:10.1136/annrheumdis-2013-205007.

Stellenbosch University https://scholar.sun.ac.za

Yamada, T., Steinz, M.M., Kenne, E. and Lanner, J.T. (2017) Muscle Weakness in Rheumatoid Arthritis: The Role of Ca2 +and Free Radical Signaling, *EBioMedicine*, 23, pp. 12–19. doi:10.1016/j.ebiom.2017.07.023.

Yang, W. and Hu, P. (2018) Skeletal muscle regeneration is modulated by inflammation, *Journal of Orthopaedic Translation*, 13, pp. 25–32. doi:10.1016/j.jot.2018.01.002.

Yin, L., Liu, M.X., Li, W., Wang, F.Y., Tang, Y.H. and Huang, C.X. (2019) Over-expression of inhibitor of differentiation 2 attenuates post- infarct cardiac fibrosis through inhibition of TGF-β1/SMAD3/HIF-1α/ Il-11 signaling pathway, *Frontiers in Pharmacology*, 10(November), pp. 1–17. doi:10.3389/fphar.2019.01349.

Yu, Z., Li, P., Zhang, M., Hannink, M., Stamler, J.S. and Yan, Z. (2008) Fiber type-specific nitric oxide protects oxidative myofibers against cachectic stimuli, *PLoS ONE*, 3(5), pp. 1–10. doi:10.1371/journal.pone.0002086.

Yue, Y., Huang, S., Wang, L., Wu, Z., Liang, M., Li, H., Lv, L., Li, W. and Wu, Z. (2020) M2b macrophages regulate cardiac fibroblast activation and alleviate cardiac fibrosis after reperfusion injury, *Circulation Journal*, 84(4), pp. 626–635. doi:10.1253/circj.CJ-19-0959.

Zeisberg, M., Hanai, J., Sugimoto, H., Mammoto, T., Charytan, D., Strutz, F. and Kalluri, R. (2003) BMP-7 counteracts TGF-β1- induced epithelial-to-mesenchymal transition and reverses chronic renal injury, *Nature Medicine*, 9(7), pp. 964–968.

Zhang, Q., Yuan, X., Li, C., Wei, W., Shen, W., Cui, Y. and Yuan, X. (2019) Macrophage depletion with clodronate-containing liposomes affects the incidence and development of rheumatoid arthritis, *Zeitschrift fur Rheumatologie*, 78, pp. 996–1003. doi:10.1007/s00393-018-0563-x.

Zhou, L., Porter, J.D., Cheng, G., Gong, B., Hatala, D.A., Merriam, A.P., Zhou, X., Rafael, J.A. and Kaminski, H.J. (2006) Temporal and spatial mRNA expression patterns of TGF-b1, 2, 3 and TbRI, II, III in skeletal muscles of mdx mice, *Neuromuscular Disorders*, 16, pp. 32–38. doi:10.1016/j.nmd.2005.09.009.

Zhu, J., Li, Y., Lu, A., Gharaibeh, B., Ma, J., Kobayashi, T., Quintero, A. and Huard, J. (2011) Follistatin improves skeletal muscle healing after injury and disease

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through an interaction with muscle regeneration, angiogenesis, and fibrosis, *Musculoskeletal Pathology*, 179(2), pp. 915–930. doi:10.1016/j.ajpath.2011.04.008.

Zou, Y., Zhang, R.Z., Sabatelli, P., Chu, M.L. and Bönnemann, C.G. (2008) Muscle interstitial fibroblasts are the main source of collagen VI synthesis in skeletal muscle: Implications for congenital muscular dystrophy types Ullrich and Bethlem, *Journal of Neuropathology and Experimental Neurology*, 67(2), pp. 144–154. doi:10.1097/nen.0b013e3181634ef7.

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# Appendices

## Appendix A – ethical approval letters

### A1 – ACUD17-00034

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**Approved Protocol**

**Date:** 26 – July – 2017

**PI Name:** Dr Ayodeji AB Oyenihi

**Protocol #:** SU-ACUD17-00034

**Title:** Potential modulators of Arthritis-related inflammation: An in vivo investigation

**Dear** Ayodeji Oyenihi, the Response to Modification, was reviewed on 19-June-2017 by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved. Please note that this clearance is only valid for a period of twelve months. Ethics clearance of protocols spanning more than one year must be renewed annually through submission of a progress report, up to a maximum of three years.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your protocol number, SU-ACUD17-00034 on any documents or correspondence with the REC: ACU concerning your research protocol. Please note that the REC: ACU has the prerogative and authority to ask further questions, seek additional information, require further modifications or monitor the conduct of your research. Any event not consistent with routine expected outcomes that results in any unexpected animal welfare issue (death, disease, or prolonged distress) or human health risks (zoonotic disease or exposure, injuries) must be reported to the committee, by creating an Adverse Event submission within the system.

We wish you the best as you conduct your research. If you have any questions or need further help, please contact the REC: ACU secretariat at wabeukes@sun.ac.za or 021 808 9003.

Sincerely, REC: ACU Secretariat

Research Ethics Committee: Animal Care and Use

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## A2 – HREC S20/01/023 (13147)

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**Approved with Stipulations**

**New Application**

06/05/2020

**Project ID**: 13147

**HREC Reference No:** S20/01/023 (PhD)

**Project Title:** The effect of chronic inflammatory disease, specifically rheumatoid arthritis, on the communication between macrophages, myoblasts and fibroblasts, in the context of skeletal muscle wasting

Dear Miss Tracey Ollewagen

The **New Application** received on **30-January-2020** was reviewed at the convened meeting for **Health Research Ethics Committee (HREC2)** on **19-February-2020** and was deferred. Response to deferral and additional information was provided **25 -March-2020** and reviewed.

The study is now approved with stipulations as of the above date.

Please note the following information about your approved research protocol:

Protocol Approval Period:**06-May-2020 – 05-May-2021**.

**The stipulations of your ethics approval are as follows:**

1. The risk of potential loss of confidentiality should be added as a risk, together with the measures taken to alleviate this risk, in both the ICF for RA patients as well as the protocol. (i.e. participant de-identification should be recognized and clearly explained in the risks sections of both documents)

Please remember to use your project ID 13147 and ethics reference number S20/01/023 (PhD) on any documents or correspondence with the HREC/UREC concerning your research protocol.

Translation of the consent document(s) to the language(s) applicable to your study participants should now be submitted to the HREC.

Please note that this decision will be ratified at the next HREC full committee meeting. HREC reserves the right to suspend approval and to request changes or clarifications from applicants. The coordinator will notify the applicant (and if applicable, the supervisor) of the changes or suspension within 1 day of receiving the notice of suspension from HREC. HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

**After Ethical Review:**

Please note you can submit your progress report through the online ethics application process, available at: https://apply.ethics.sun.ac.za and the application should be submitted to the Committee before the year has expired. Please see Forms and Instructions on our HREC website for guidance on how to submit a progress report.

The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

**Provincial and City of Cape Town Approval**

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: https://www.westerncape.gov.za/general-publication/health-research-approval-process. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and instructions, please visit: Forms and Instructions on our HREC website (www.sun.ac.za/healthresearchethics)

If you have any questions or need further assistance, please contact the HREC office at 021 938 9677.

Yours sincerely,

Mrs. Brightness Nxumalo

HREC 2 Coordinator

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## A3 – REC:BEE-2020-18524

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**Approval Letter**

21 January 2021

**PI:** Miss Tracey Ollewagen

**REC: BEE Reference #:** BEE-2020-18524

**Title:** The effect of chronic inflammatory disease on cellular communication

Dear Miss Tracey Ollewagen

Your response to modifications with reference number #BEE-2020-18524 was reviewed by the Research Ethics Committee: Biosafety and Environmental Ethics via committee review procedures and was approved. Please note that this clearance is only valid for a period of twelve months. Ethics clearance of protocols spanning more than one year must be renewed annually through submission of a progress report, up to a maximum of three years.

Approval Date: **21 January 2021- 20 January 2022**

**Note:** Your progress report is due one month prior to the expiry date of this project approval.

Please remember to use your REC: BEE reference number: # BEE-2020-18524 on any documents or correspondence with the REC: BEE concerning your research protocol.

If you have any questions or need further help, please contact the REC: BEE office at 021 808 9003.

Visit the Division for Research Developments website www.sun.ac.za/research for documentation on REC: BEE policy and procedures.

Sincerely,

Mr Winston Beukes

Coordinator: Research Ethics (Biosafety)

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**Appendix B: Patient information leaflet and consent form**

# PARTICIPANT INFORMATION LEAFLET

**TITLE OF THE RESEARCH PROJECT: The effect of chronic inflammation on cellular communication in skeletal muscle**

REFERENCE NUMBER: S20/01/023

PRINCIPAL INVESTIGATOR: Miss Tracey Ollewagen

ADDRESS: Department of Physiological Sciences, Mike De Vries Building,

Cnr Merriman and Bosman streets, Stellenbosch

CONTACT NUMBER: 021 808 3424

We would like to invite you to participate in a research project that aims to investigate the effect that chronic inflammatory diseases, such as rheumatoid arthritis, have on skeletal muscle. We aim to investigate the mechanisms behind muscle wasting in these chronic diseases in order to develop better treatment options.

Please take some time to read the information presented here, which will explain the details of this project and contact me if you require further explanation or clarification of any aspect of the study. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever.

This study has been approved by the Health Research Ethics Committee at Stellenbosch University. The study will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, the South African Guidelines for Good Clinical Practice (2006), the Medical Research Council (MRC) Ethical Guidelines for Research (2002), and the Department of Health Ethics in Health Research: Principles, Processes and Studies (2015).

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What is this research study about?

Research has shown that chronic inflammation leads to a condition in which the muscle is degraded and undergoes wasting; in the context of rheumatoid arthritis, this is called rheumatoid cachexia. This muscle wasting often occurs even before RA is diagnosed but continues throughout disease because of the inflammatory cells circulating the body. This often leads to reduced quality of life. Unfortunately, this affects the quality of life of a large number of patients.

The study you are being recruited for will use blood and/or synovial fluid from patients with rheumatoid arthritis, as well as healthy controls. Synovial fluid is the liquid found in the joints that reduce the friction between the bones, such as in the knee. Excessive synovial fluid leads to swelling, pain and discomfort in the joint and is frequently removed by doctors to reduce the pressure on the joint. The blood and synovial fluid will be used to simulate RA conditions at cellular level in our laboratory. We aim to use these fluids to treat various healthy cells from skeletal muscle to determine the changes that the inflammatory components within the blood and synovial fluid have on the skeletal muscle system. Skeletal muscle is the most common of the muscle types and is considered to be the muscles that allow voluntary movement. Once we have a better understanding of the changes that occur as a result of the inflammation, we will be able to develop better treatment strategies to improve the quality of life in patients suffering from chronic inflammatory diseases, as well as potentially assist those patients who are resistant to the current treatments. This indicates the importance of this kind of study.

The study in which you will be participating will be conducted solely in the rooms of your clinician during a routine visit. You will not be required to have any additional doctors' visits to contribute to this research as all blood and/or synovial fluid withdrawal will be done during your usual consultations with the doctor. During the visit to your rheumatologist, the doctor or nurse will draw blood from the antecubital (arm) vein and/or synovial fluid from the affected joint as they would do during normal testing session, taking an additional 5 tubes of blood for the researcher. You will not be required to take any additional medication or perform any additional activities during this study.

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Why are you invited to participate in this study?

We invite you participate in the study due to your medical status as a patient with rheumatoid arthritis, or a healthy control. Within the blood and synovial fluid are inflammatory cells and molecules which circulate the body and affect various different tissues. This is elevated in patients with chronic inflammation. This means that the disease does not necessarily affect a single part of your body. A number of patients with chronic inflammation suffer from muscle wasting as a result of these inflammatory components, often affecting their quality of life. We invite you to participate in this study so we can investigate the mechanisms behind this muscle wasting more comprehensively and begin the development of treatment strategies to reduce this muscle wasting and improve quality of life of patients. We also require healthy individuals so we can compare the effects to a healthy population.

What will your responsibilities be?

Your main responsibility in this study is to be honest with the doctor regarding treatment and medications currently being taken. We need to know all medications taken as this may affect the inflammatory profile in your blood and this will determine the quality of the data we are able to generate and the interpretations we make. For healthy controls, we require that you are not currently on any anti-inflammatory medication or treatment. You will be required to give 5 tubes of blood and/or the synovial fluid taken to relieve the joint. This may be additional to the blood/synovial fluid the doctor may take for testing. Blood and synovial fluid will only be withdrawn if you are scheduled for blood testing or withdrawal of synovial fluid for therapeutic purposes.

Are there any risks involved in taking part in this research?

Participation in this study will not add to the minimal risks normally involved when visiting your doctor. For RA patients, blood or synovial fluid will only be withdrawn if you are scheduled for a routine/therapeutic withdrawal. Therefore, the only risks are those involved in a normal draw including bruising at the draw site. For healthy

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patients, blood will be taken as per WPBTS protocol and the only risks involved is bruising at the draw site.

Who has access to your medical records?

All information will be collected by the doctor and given a patient code, therefore no patient identities will be shared with the researcher and all patients will remain anonymous. All medical information and questions shared will be given using the patient code. The only personal information we would require from the doctor, is your age, weight and inflammatory disease status, as well as recent (3 month) treatment history.

Are there any costs involved in the study?

There are no costs involved in the study as all samples will be taken during a regular doctors visit and all analyses required for the research will be paid from a research grant held by the investigators.

If you have any additional questions or encounter any problems, you may contact the researchers – Miss Tracey Ollewagen on 021 808 3424

You can also phone the Health Research Ethics Committee at 021 938 9677/9819 if you have any additional questions or if you have a complaint.

If you are willing to participate in this study, please sign the attached Declaration of Consent and give it to your doctor

Yours sincerely

Tracey Ollewagen/Prof Carine Smith

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Declaration by participant

**By signing below, I agree to take part in a research study entitled: The effect of chronic inflammation on cellular communication in skeletal muscle**

.

I declare that:

- I have read the attached information leaflet and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…..…….. On (*date*) ……...…….. 2020.

**----------**

**Signature of participant**

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## Appendix C: Medical questionnaire form

Medical data collection form

Patient ID code: _____

Date of birth: _____

Weight: _____

Height: _____

Waist circumference: _____

Ethnicity: _____

Gender: Male / Female

Medical condition: Rheumatoid arthritis / Healthy control

RA classification/progression status: Treatment naïve / Treatment non-responding

Major joint affected: _____

Number of years diagnosed: _____

Current treatment:

_____

_____

If treatment naïve, time period since last treatment:

_____

Any other conditions:

_____

_____

Current other medications:

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_____

_____

_____

Experience any obvious loss of muscle mass or loss of muscle strength: Yes / No

Current smoker: Yes / No

Previous smoker (last 10 years): Yes / No

*If you have any difficulties filling in this form, the doctor or liaison will be ready to assist

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## Appendix D: Monocyte isolation protocol

Isolation of human monocytes from donor whole blood by double gradient centrifugation

1. Carefully disinfect the EDTA vacutainers containing the peripheral whole blood and transfer the contents to sterile plastic tubes. Do not mix blood from different donors.
2. For 2 x 8mL EDTA vacutainers fill two 15 ml tubes with 4 ml Ficoll/Histopaque solution (1.077 g/ml) each. The Ficoll should be at room temperature for the preparation.
3. Layer 8 ml whole blood on top of the Ficoll solution for the first density gradient. Be careful to do this slowly and carefully in order to prevent mixing both layers.
   **Note**: Maintain Ficoll:blood ratio at 1:1-2
4. Centrifuge at 400 x g without brake for 30 min at room temperature.
5. Add 1 mL of 1 mM PBS-EDTA (1x PBS and 0.5 M EDTA) into two new 15 mL tubes. This will ensure continued EDTA exposure and prevent clumping.
6. For each gradient collect the white ring of peripheral blood mononuclear cells (PBMCs) which is located between the two phases with a plastic Pasteur pipette and transfer to the new 15 ml tubes.
7. Fill each tube with PBS-EDTA up to the 12 mL mark.
8. Centrifuge at 300 x g for 10 min without brake at room temperature.
9. Aspirate supernatant and wash pellet again with appropriate amount of PBS-EDTA.
10. For each donor pool the pellets in 6 ml RPMI-1640 **without** phenol red + 10% FBS.
11. Prepare the iso-osmotic Percoll solution for the second density gradient: For two donors mix 6.939 ml Percoll solution (density: 1.131 g/ml) in a 15 ml tube with 0.561 ml 10x PBS. Then transfer 6.9 ml of this solution to a new 15 ml tube and add 8.1 ml RPMI-1640 **with** phenol red + 10% FBS to obtain a 46% iso-osmotic Percoll solution. The Percoll should be at room temperature for the preparation.
12. For each donor transfer 7.3 ml of the prepared Percoll solution to a 15 ml tube and layer the PBMC solution prepared in step 10 on top of the Percoll

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solution. Be careful to do this very slowly and carefully, both layers tend to mix easily. If done correctly the two phases can be distinguished due to their difference in colour.

13. Centrifuge at 550 x g without brake for 30 min at room temperature.
14. For each gradient collect the white ring of monocytes which is located between the two phases with a plastic Pasteur pipette and transfer to a new 15 ml tube.
15. Fill each tube with the appropriate amount of PBS-EDTA.
16. Centrifuge at 400 x g for 10 min without brake at room temperature.
17. Aspirate the supernatant and resuspend the pellets in the needed amount of Complete Monocyte media or FBS to seed or freeze cells, respectively.

Pre-differentiation of Monocytes into M1 and M2 Macrophages

1. Seed fresh (never before frozen) monocytes in polystyrene culture plates/dishes or pre-coated plates. Note: Monocytes struggle to adhere to glass.
2. For M1 type: Treat with 50ng/ml GM-CSF directly after seeding.
   For M2 type: Treat with 50ng/ml M-CSF directly after seeding.
3. Allow to adhere for 24h.
4. Aspirate media and wash with warm 1x PBS.
5. Add complete monocyte media and treat with 50 ng/mL GM-CSF or M-CSF.
6. Change media and wash with warm PBS every 3$^{rd}$ day.
7. Do not culture cells for more than 10 days in total.
8. **Polarize cells.** M1: Pre-treat cells with 50 ng/mL LPS and 20 ng/mL IFN-γ for 24h. M2: Pre-treat cells with 20 ng/mL IL-10, IL-4 and TGF-β for 24h.

Complete Monocyte Media

RPMI 1640 (advanced RPMI also works).

10% Human Serum from AB patient (CAT# H4522 Sigma-Aldrich)

1% Penicillin-Streptomycin (100 U/mL)

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50 ng/mL GM-CSF (CAT# SRP3050 Sigma-Aldrich)

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## Appendix E: BMP-7 single cell experiments

### Purpose of the single cell culture experiments

The aim of the pilot single cell culture experiments were to determine the optimal BMP-7 concentration to alter the inflammatory phenotype from a M1 to a M2 phenotype, to improve muscle growth and proliferation without changing the cellular phenotype to an osteogenic profile, and to reduce fibrosis indicators.

### Methods

Primary blood-derived monocytes were differentiated and polarised to a M1 phenotype before being treated with incremental concentrations of BMP-7 (0, 50, 250, 500, 750 ng/ml) in FBS-containing media for 48 hours. Flow cytometry was performed to determine the percentage of M2c macrophages in the culture through the identification of extracellular markers (CD14, CD163, CD206, viability) and intracellular markers (IL-10, Arginase-1).

Primary myoblasts were plated in 6-well plates with FBS-containing media and treated with incremental concentrations of BMP-7 for 48 hours. Images were taken on the Olympus bright field microscope at 40x and 100x magnification for the assessment of area fraction and cell size. Protein was extracted for analysis of BMP-7 and its receptor, pSmad1/5/8, and muscle-specific growth factors (TGF-β, MyoD, Pax7, Id2) and Runx-2 (osteoblast marker). Scratch analysis was performed on myoblasts in 24-well plates treated with BMP-7 to determine migration ability.

Primary fibroblasts were plated in 6-well plates with FBS-containing media and treated with incremental concentrations of BMP-7 for 48 hours. Images were taken on the Olympus bright field microscope at 40x and 100x magnification for the assessment of area fraction. Protein was extracted for analysis of BMP-7, pSmad1/5/8, and fibroblast-specific growth factors (TGF-β, Id2, α-SMA).

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## Results

Flow cytometry analysis demonstrated that M1 macrophage monocultures treated with BMP-7 did not reduce the viability of cells (Figure E1a). Flow cytometry of cells stained with CD14 indicated that between 70-80% of cells were successfully differentiated to macrophages from monocytes. There were no significant differences in differentiation success between the treatment dose groups (Figure E1b). Treatment with 500 and 750 ng/ml of BMP-7 significantly increased the percentage of cells polarized to an anti-inflammatory M2c phenotype ($p<0.05$; Figure E1c), as determined by concurrent positive staining with CD14, CD163, CD206, IL-10 and Arginase-1.

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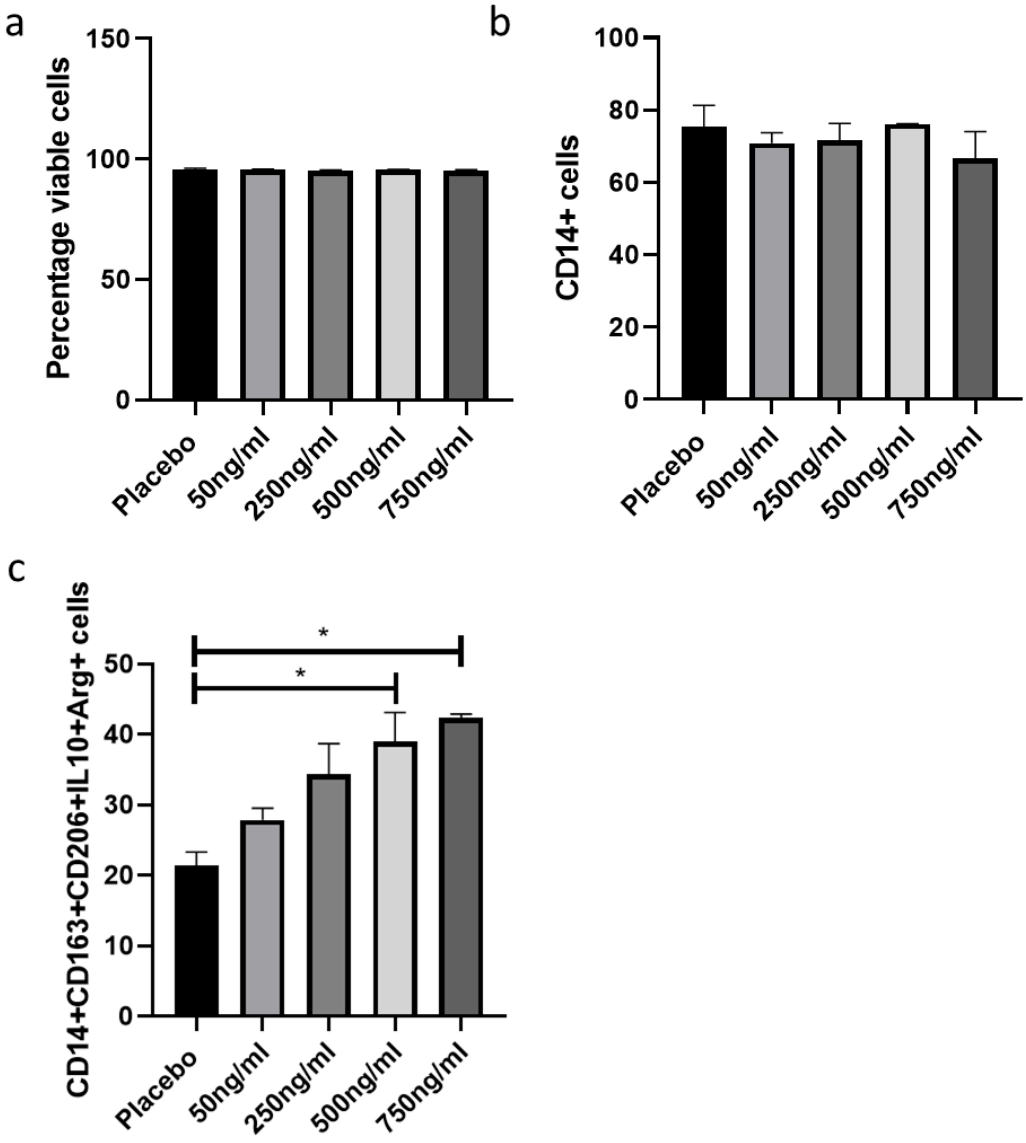


*Figure E1: Flow cytometric analysis of M1 macrophages treated with BMP-7 for 48 hours. a) Percentage of viable cells; b) Percentage of CD14+ cells; c) Percentage of M2c macrophages as indicated by positive signal for CD14, CD163, CD206, IL-10 and Arginase-1. n=2 per group. Statistical analysis: One-way ANOVA with Tukey's multiple comparisons test. * = p<0.05. BMP-7 = bone morphogenic protein-7.*

Analysis of myoblast cell images was used to assess area fraction, cell size and migration ability with a scratch assay. Area fraction of the myoblasts did not differ significantly with increasing concentrations of BMP-7 (Figure E2a). However, cell size did increase following treatment with 750 ng/ml of BMP-7 ($p<0.05$; Figure E2b).

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There were no significant differences in migration ability between the dosages, except at 1 hour post-scratch where 50 ng/ml demonstrated reduced migration ability ($p<0.05$; Figure E2c).

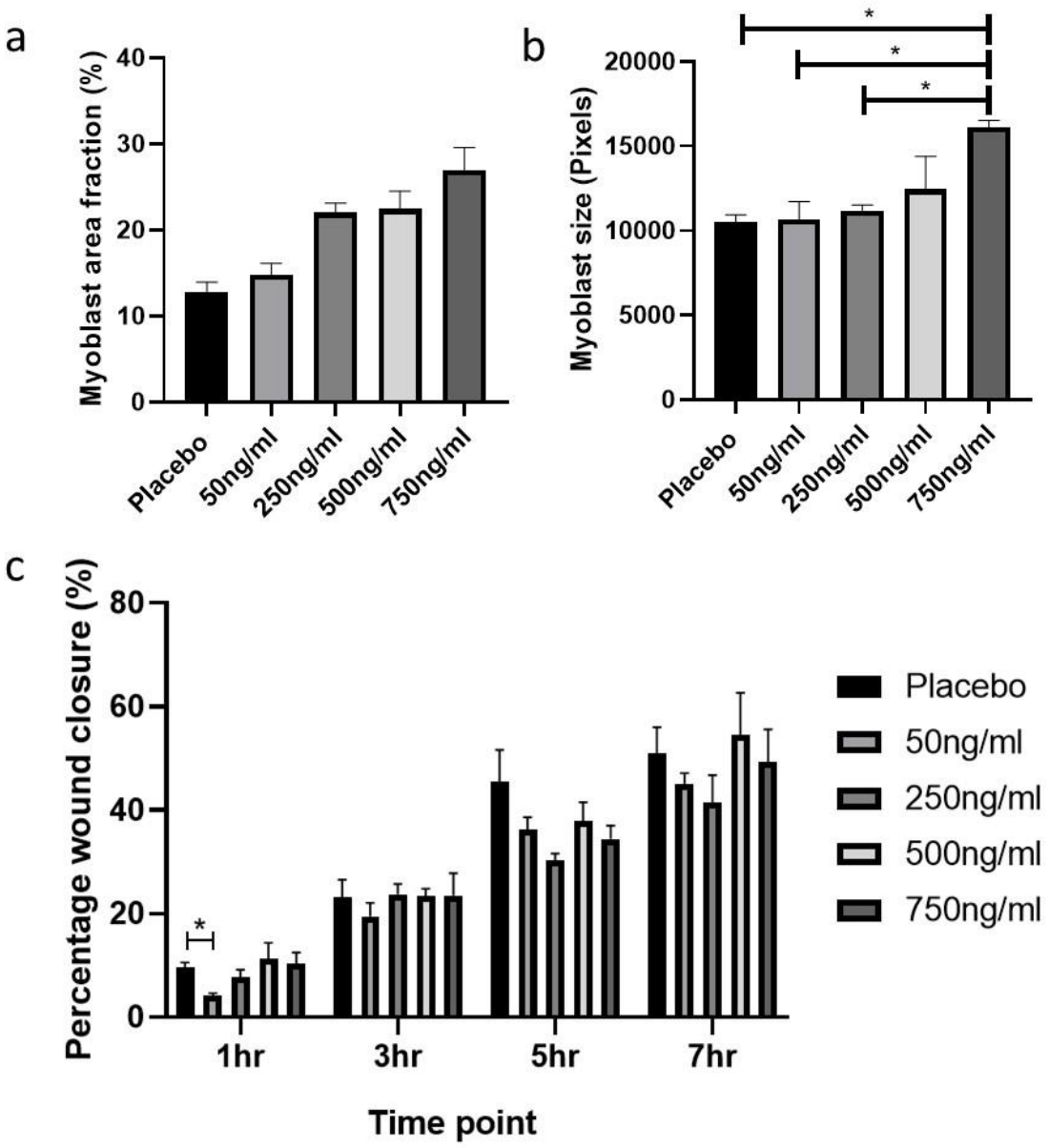


*Figure E2: Myoblast response to 48 hour BMP-7 treatment. a) Myoblast area fraction; b) Myoblast cell size; c) Percentage wound closure over 7 hours following scratch assay. n=3 per group. Statistical analysis: one-way ANOVA with Tukey's multiple comparisons test (a); Kruskal-Wallis test with Dunn's multiple comparisons test (b); and two-way ANOVA with Tukey's multiple comparisons test (c). * = p<0.05.*

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Western blot analysis of myoblasts demonstrated no significant effect of BMP-7 treatment on BMP-7, pSmad1/5/8 receptor, TGF-β, MyoD, and Pax7 concentration (Figure E3a-e). Id2 protein concentration was significantly greater than the placebo following treatment with 250 ng/ml of BMP-7 ($p<0.01$; Figure E3f). Runx2, a marker of bone differentiation, was significantly lower than day 3 and day 7 differentiating chondroblast controls in the placebo, 50, 250 and 500 ng/ml groups ($p<0.05$; Figure E3g), indicating no bone differentiation as a result of BMP-7 treatment.

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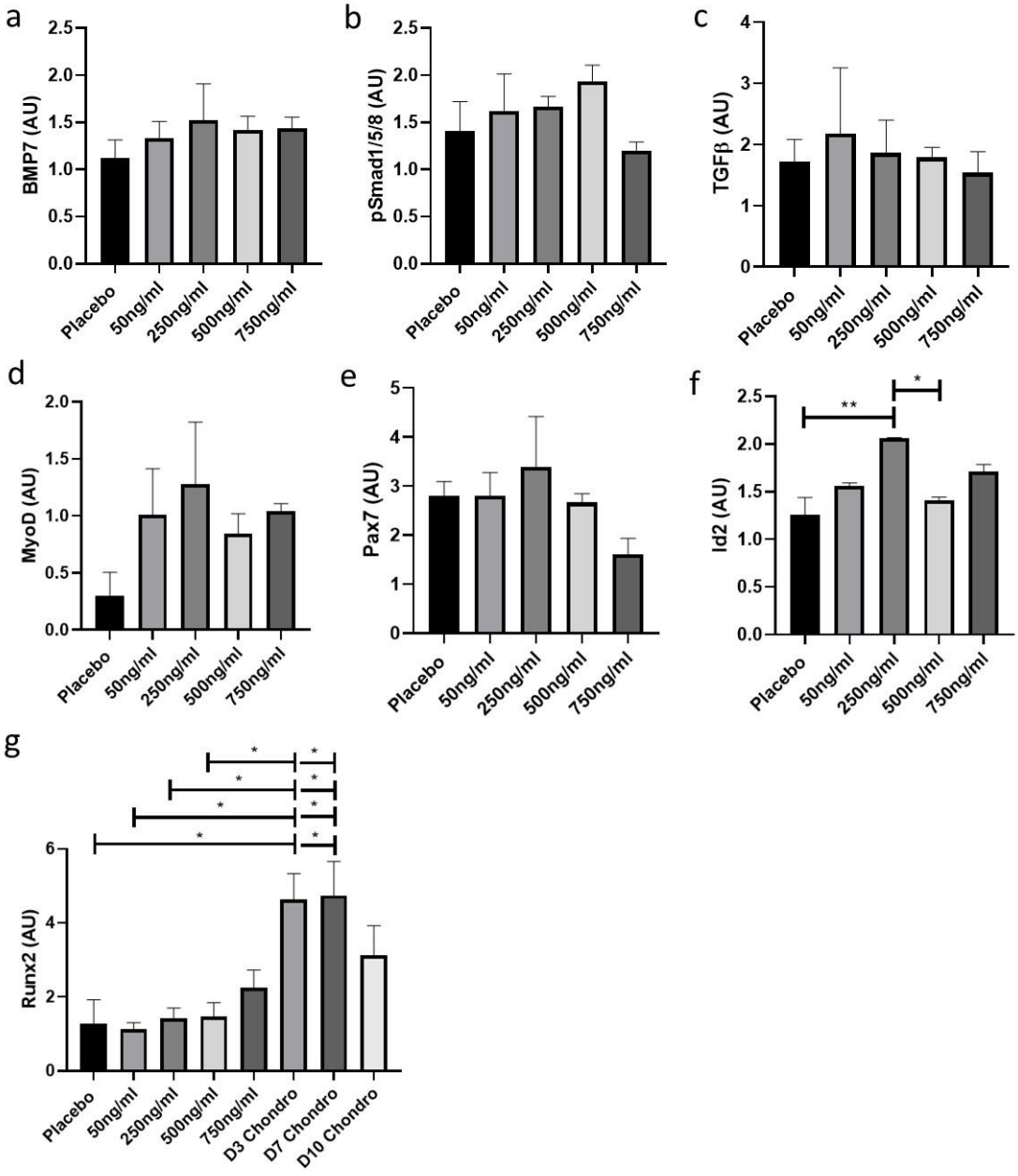


*Figure E37: Western blot analysis of myoblast protein following BMP-7 treatment for 48 hours. a) BMP-7; b) pSmad1/5/8; c) TGF-β; d) MyoD; e) Pax7; f) Id2; g) Runx2 (with differentiation chondroblasts as a control). n=3 per group. Statistical analysis: One-way ANOVA with Tukey's multiple comparisons test. * = p<0.05; ** = p<0.01. BMP-7 = bone morphogenic protein-7; TGF-β = transforming growth factor-β; Pax7 = paired box 7; Id2 = inhibitor of differentiation 2; Runx2 = runt-related transcription factor-2.*

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Image analysis of fibroblasts indicated a significant difference in area fraction between the 50 ng/ml and 750 ng/ml BMP-7-treated cells ($p<0.05$; Figure E4a). Western blot analysis demonstrated no significant effect of BMP-7 treatment on BMP-7, pSmad1/5/8, TGF-β, and α-SMA (Figure E4b-d, f). However, Id2 protein concentration was significantly elevated following treatment with 750 ng/ml of BMP-7 ($p<0.05$; Figure E4e).

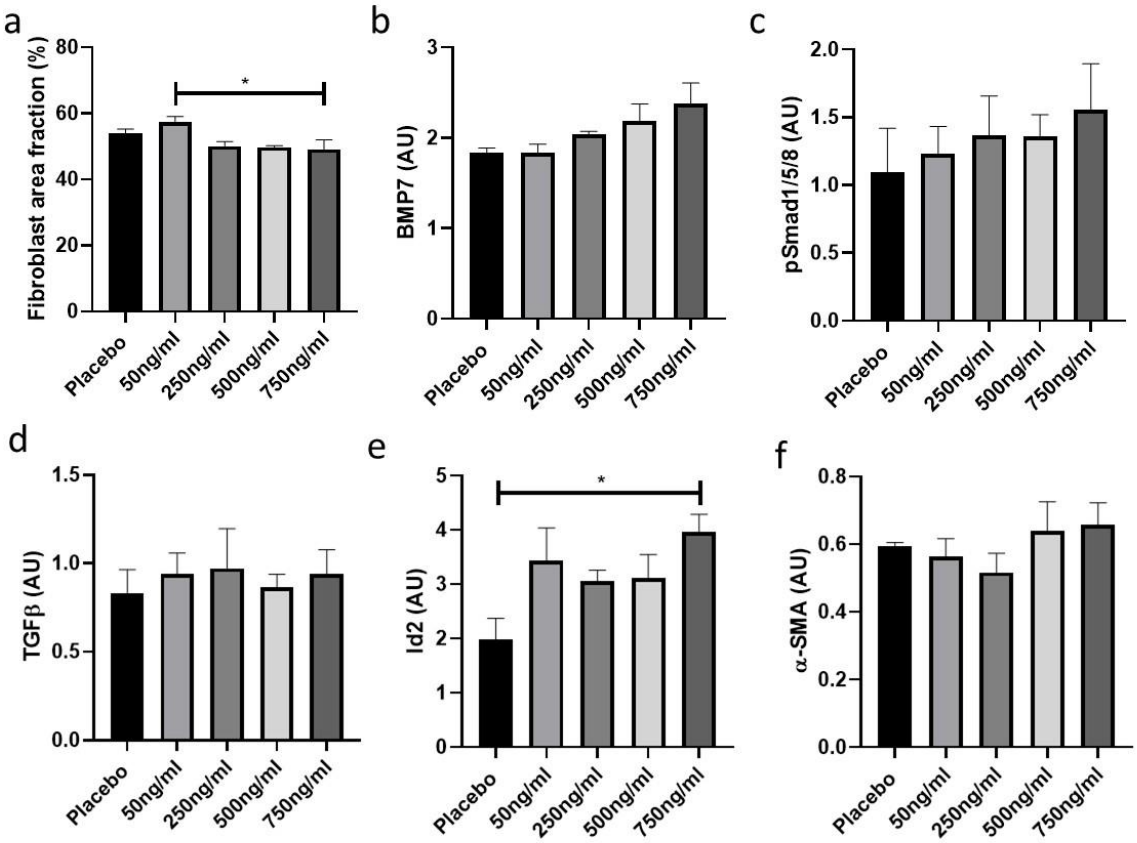


*Figure E4: Fibroblast area fraction and Western blot analysis of fibroblast protein following BMP-7 treatment for 48 hours. a) area fraction; b) BMP-7; c) pSmad1/5/8; d) TGF-β; e) Id2; f) α-SMA. n=3 per group. Statistical analysis: One-way ANOVA with Tukey's multiple comparisons test. * = p<0.05. BMP-7 = bone morphogenic protein-7; TGF-β = transforming growth factor-β; Id2 = inhibitor of differentiation 2; α-SMA = α-smooth muscle actin.*

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## Conclusion

The data suggests that the optimal BMP-7 dose to alter macrophages to a beneficial M2 phenotype, improve muscle growth without altering the phenotype to that of osteoblasts, and reduce fibroblast growth is 750 ng/ml.

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## Appendix F: Flow cytometry protocol

### F1 Flow cytometric analysis method

Following treatment of macrophages with BMP-7 for 48 hours, cells were analysed for macrophage phenotype with flow cytometry. Cells were harvested with Accutase and transferred to 5 ml FACS tubes. After adding 1 ml PBS, cells were centrifuged for 5 minutes at 400 x g. 50 µl of Fc block diluted at 1:100 was added to the cell suspension and incubated for 20 minutes at 4$^{o}$C, followed by centrifugation for 10 minutes at 250 x g. Cells were re-suspended in FACS buffer with surface staining markers (CD14, CD163, CD206, viability dye) at their pre-optimized concentrations. Cells were incubated for 30 minutes at 4$^{o}$C in the dark followed by washing with FACS buffer and centrifuged for 10 minutes at 250 x g. All washes in the remainder of the protocol were repeated in the same manner. Cells were re-suspended in fixation buffer (4% PFA) and incubated at 4$^{o}$C for 20 minutes in the dark. Following the wash step, cells were re-suspended in permeabilization buffer (0.1% saponin) and incubated for 15 minutes at 4$^{o}$C in the dark, followed by another wash step. Cells were re-suspended in FACS buffer with the appropriate volume of intracellular staining markers (IL-10, Arginase-1) and incubated for 30 minutes at 4$^{o}$C in the dark. After washing, cells were re-suspended in FACS buffer for analysis on the FACS Melody.

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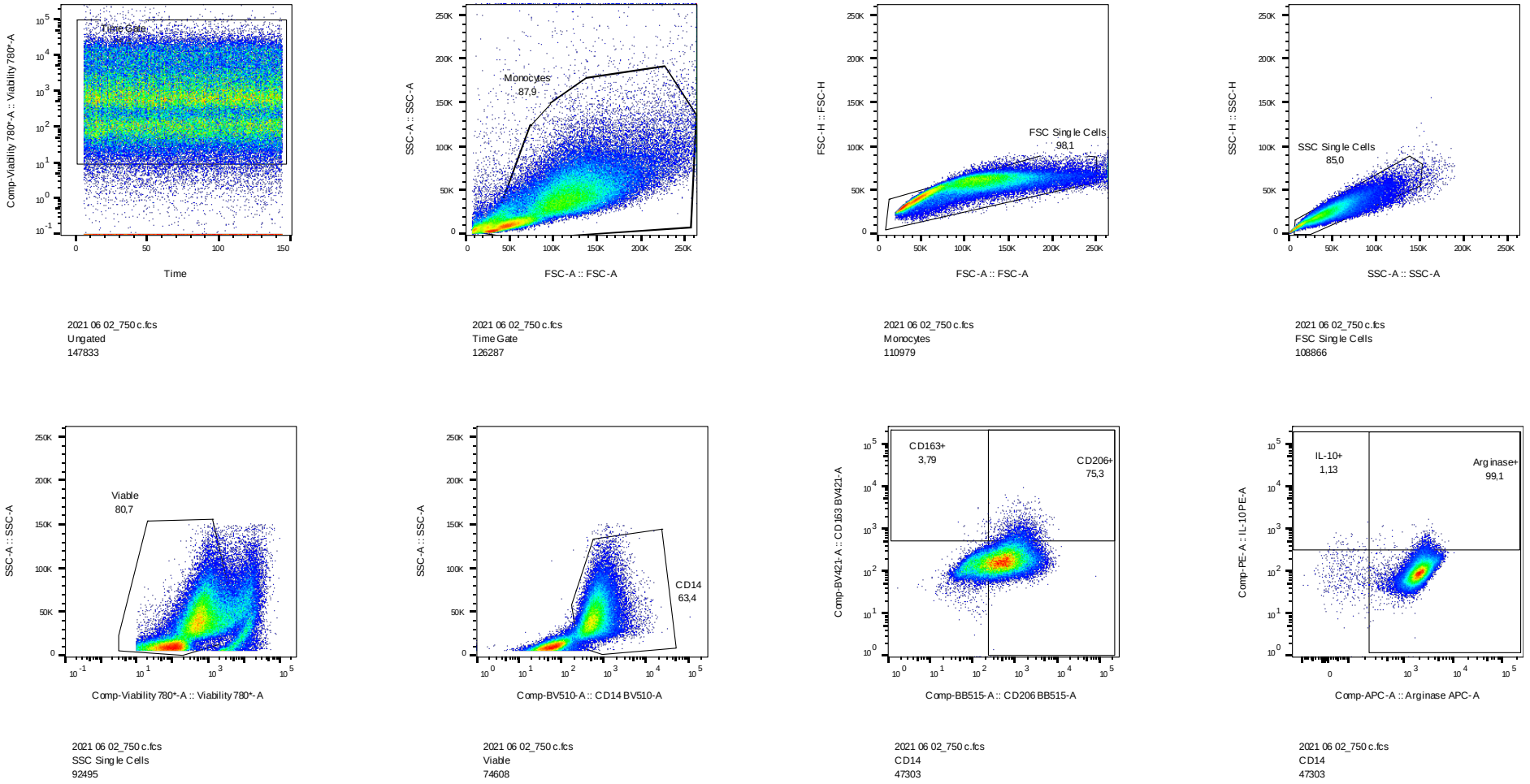


*Figure F1: Representative flow cytometry scatter plots illustrating M2 macrophage gating strategy.*

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## F2 Instrument set-up

The BD FACS Melody flow cytometer (BD Biosciences, USA), housed in the Central Analytic Facilities Fluorescent Imaging Unit, Stellenbosch University, was employed for the analysis of macrophage samples.

The following table displays the filters (longpass and bandpass), detectors and voltage settings employed in the macrophage assay:

*Table F2: Instrument parameters for macrophage assay by flow cytometry*

| Laser | LP filter | BP filter | Parameter | Amplification |
|---|---|---|---|---|
| 488 nm | n/a | 488/15 | SSC-A | Linear |
| | 507 | 527/32 | BD Horizon BB515 – CD206 | Log |
| | 560 | 586/42 | PE – IL-10 | Log |
| 640 nm | n/a | 660/10 | APC – Argianse-1 | Log |
| | 752 | 783/56 | Viability Stain 780 | Log |
| 405 nm | n/a | 448/4 | BD Horizon BV421 – CD163 | Log |
| | 500 | 528/45 | BD Horizon BV510 – CD14 | Log |

## F3 Antibody information and titration protocol for platelet assay

The characteristics of all antibodies utilized in the macrophage analysis are summarised in Table F2.

*Table F3: Macrophage assay antibody characteristics*

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| Marker | Host | Species reactivity | Fluorochrome | Company | Catalogue number |
|---|---|---|---|---|---|
| Viability dye | n/a | n/a | 780 | BD Bioscience | 565388 |
| CD14 | Mouse | Human | BV510 | BD Bioscience | 563079 |
| CD206 | Mouse | Human | BB515 | BD Bioscience | 564668 |
| CD163 | Mouse | Human | BV421 | BD Bioscience | 562643 |
| IL-10 | Rat | Human | PE | BD Bioscience | 559330 |
| Arginase-1 | Rat | Human | APC | Invitrogen | 17-3697-82 |

To optimise antibody concentrations for macrophage flow cytometry, antibody titrations were performed on isolated, cultured monocytes polarized to either M1 (viability and CD14) or M2 phenotype (viability, CD14, CD163, CD206, IL-10, arginase-1). This ensured that all markers of interest would be expressed. Antibodies were diluted according to the following table:

*Table F4: Macrophage assay antibody titrations*

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| Marker | Volume (µl) | Recommended (per sample) | Dilution A | Dilution B | Dilution C | Dilution D |
|---|---|---|---|---|---|---|
| Viability | 100 | 0.1 µl | 0.1 µl* | 0.05 µl | 0.025 µl | 0.0125 µl |
| CD14 | 100 | 5 µl | 5 µl* | 2.5 µl | 1.25 µl | 0.625 µl |
| CD163 | 100 | 5 µl | 5 µl* | 2.5µl | 1.25 µl | 0.625 µl |
| CD206 | 100 | 5 µl | 5 µl* | 2.5 µl | 1.25 µl | 0.625 µl |
| IL-10 | 100 | 20 µl | 20 µl* | 10 µl | 5 µl | 2.5 µl |
| Arginase-1 | 100 | 2 µl | 2 µl* | 1 µl | 0.5 µl | 0.25 µl |

* Optimal concentrations determined by flow cytometry.