MECHANISMS UNDERLYING THE DEVELOPMENT OF WEAKNESS IN IDIOPATHIC INFLAMMATORY MYOPATHIES: AN IN VITRO SINGLE MUSCLE FIBRE CONTRACTILITY STUDY.

by

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LIST OF ABBREVIATIONS

CS	citrate synthase
CSA	cross-sectional area
DM	dermatomyositis
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FSHD	facioscapulohumeral muscular dystrophy
HMGB1	high mobility group box protein 1
IHC	immunohistochemistry
IL	interleukin
IIMs	idiopathic inflammatory myopathies
LDH	lactate dehydrogenase
Mg^{2+}	magnesium
MHC	major histocompatibility complex
MRC	Medical Research Council
MyHC	myosin heavy chain
MyLC	myosin light chain
NADH	nicotinamide adenine dinucleotide
NAM	necrotising autoimmune myopathy
NO	nitrogen oxide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PFK	phosphofructokinase
PM	polymyositis
P_0	maximum force
P_r	relative force
ROS	reactive oxygen species
SD	standard deviation
SDS	sodium dodecyl sulphate
SF	specific force
skMLCK	skeletal muscle myosin light chain kinase
SR	sarcoplasmic reticulum
TLR4	toll like receptor 4

TNF	tumour necrosis factor
Tn	troponin
TnC	Ca^{2+}-binding troponin
TnI	inhibitory troponin
TnT	tropomyosin-binding troponin
V_{max}	maximum shortening velocity
V_0	unloaded shortening velocity
3-HAD	3-hydroxyacyl-CoA dehydrogenase

LIST OF UNITS OF MEASUREMENT

g/l	grams per litre
kN	kilonewtons
M	molar (mol per litre)
m^2	square metre
µl	microliter
$µm^2$	square micrometre
mg	milligrams
ml	millilitres
mM	millimolar
mN	millinewtons
nm	newtons
nm	nanometres
U	units

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ABSTRACT

Introduction: Polymyositis (PM), dermatomyositis (DM) and necrotising autoimmune myopathy (NAM) form part of the spectrum of idiopathic inflammatory myopathies (IIMs). Although the pathogenic mechanisms are different, the unifying feature is that of weakness caused, in some way or another, by an inflammatory attack on muscle. The mechanism by which weakness develops is still unclear, but experimental animal data suggest that dysfunction of the contractile apparatus might contribute to muscle weakness in these conditions. This study investigated the contractile function of single muscle fibres from patients with IIMs *in vitro*. *Methods*: Muscle biopsies obtained from patients with IIMs and healthy controls were dissected and chemically permeabilised. Single muscle fibres were dissected out and subjected to contractility measurement based on standard protocols utilising a permeabilised single fibre system. Specific force (SF; maximum force normalised to cross-sectional area), was calculated for each fibre and compared between the two groups. In addition, maximum shortening velocity and power output were assessed in some of the fibres, and calcium sensitivity in the rest. The myosin heavy chain composition of each fibre was determined by means of gel electrophoresis. *Results:* A total of 178 fibres from IIM cases and 174 fibres from controls were studied. Specific (normalised) force was 23%, 24% and 29% lower in the IIM group for all fibre types combined, type I fibres, and type IIa fibres, respectively. Shortening velocity and maximum power output were significantly higher in the IIM group for both type I and IIa fibres, compared to controls, while calcium sensitivity was higher in type IIa fibres from IIM cases than controls. *Discussion:* The findings from this study suggest that weakness in IIMs may, at least in part, be caused by dysfunction of the contractile apparatus leading to impaired contractile force. The higher shortening velocity, power output and calcium sensitivity in fibres from IIM cases probably represents compensatory mechanisms. Although the mechanism by which contractile function is affected has not been investigated, animal studies suggest a role for TNF-α. The findings of this study provide a basis for further investigation into the mechanisms underlying weakness in IIMs.

CHAPTER ONE

1 PATHOGENIC MECHANISMS OF MUSCLE WEAKNESS IN IDIOPATHIC INFLAMMATORY MYOPATHIES: CURRENT CONCEPTS

1.1 Introduction and background

1.1.1 *Normal skeletal muscle*

1.1.1.1 *Structure of normal skeletal muscle*

Skeletal muscles are organs that consist of different integrated components, namely muscle cells (commonly referred to as muscle fibres due to their cylindrical structure), nerve fibres, blood vessels and various layers of connective tissue. The connective tissue layers allow a muscle to maintain structural integrity while contracting, separate it from surrounding tissues, and compartmentalise the muscle fibres within the muscle. Three different connective tissue layers can be identified within each muscle (*Figure 1*), namely the epimysium (surrounds the whole muscle), the perimysium (organises the muscle fibres into groups called fascicles), and the endomysium (encases individual muscle fibres).

Muscle fibres are the individual functional units of skeletal muscles. Each fibre consists of a sarcolemma (cell membrane) surrounding the sarcoplasm (the cytoplasm of muscle cells). Within the cytoplasm are myofibrils, each consisting of strands of filaments, arranged into repeating units called sarcomeres (Figure 2). Surrounding each myofibril is the sarcoplasmic reticulum (SR), which is the source of intracellular

calcium (Ca^{2+}) required for contraction. In order for an action potential to rapidly lead to contraction, the sarcolemma is in close contact with the SR by means of invaginations called T-tubules.

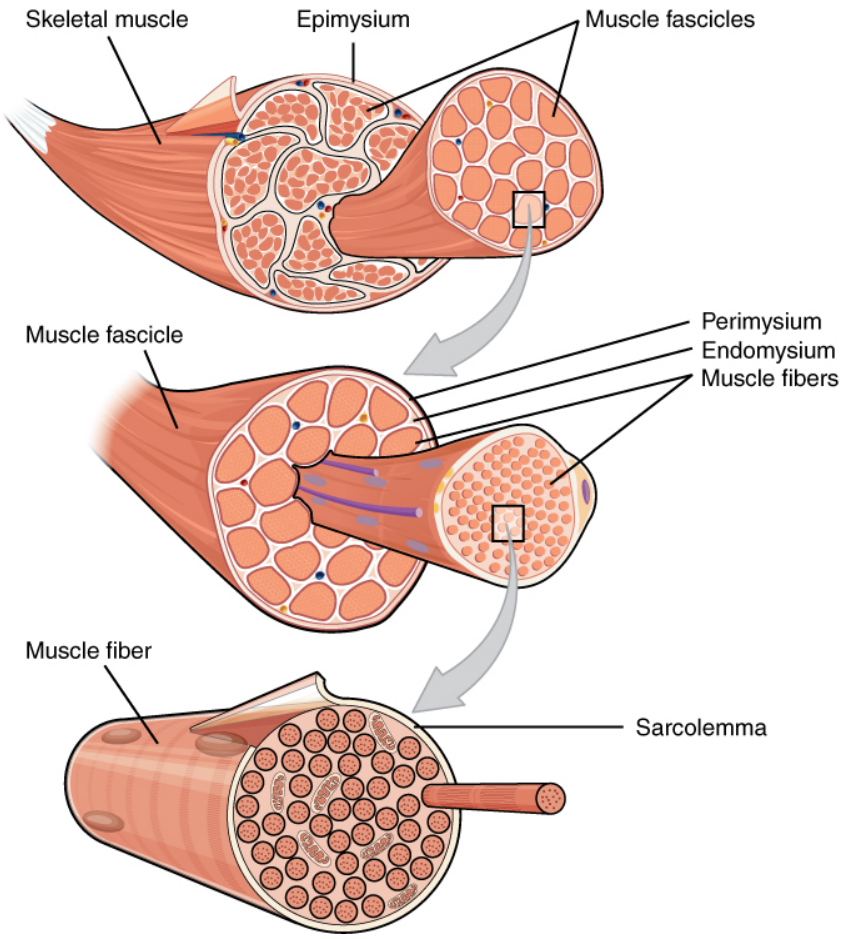

Figure 1. Structural organisation of skeletal muscles.
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The sarcomere forms the functional unit of the myofibril and are the units that are responsible for the striated appearance of skeletal muscle fibres, which can best be seen with electron microscopy (Figure 3). Each sarcomere contains a three-dimensional arrangement of thick and thin filaments. Thin filaments consist primarily of actin, troponin and tropomyosin (visible as the I-band, Figure 3), while thick

filaments consist of type II myosin (visible as the A-band, Figure 3). Individual sarcomeres are bordered on each side by Z-disks (also called Z-lines), to which actin filaments are anchored (figure 4). Other muscle proteins include alpha-actinin (an actin-binding protein and major component of the Z-line), titin (which runs from the M-line to the Z-line, functions as a molecular spring and is responsible for passive force reduction) and costameres, which are regions associated with the sarcolemma of skeletal muscles that aid in transmitting force from the contractile apparatus to the extracellular matrix.

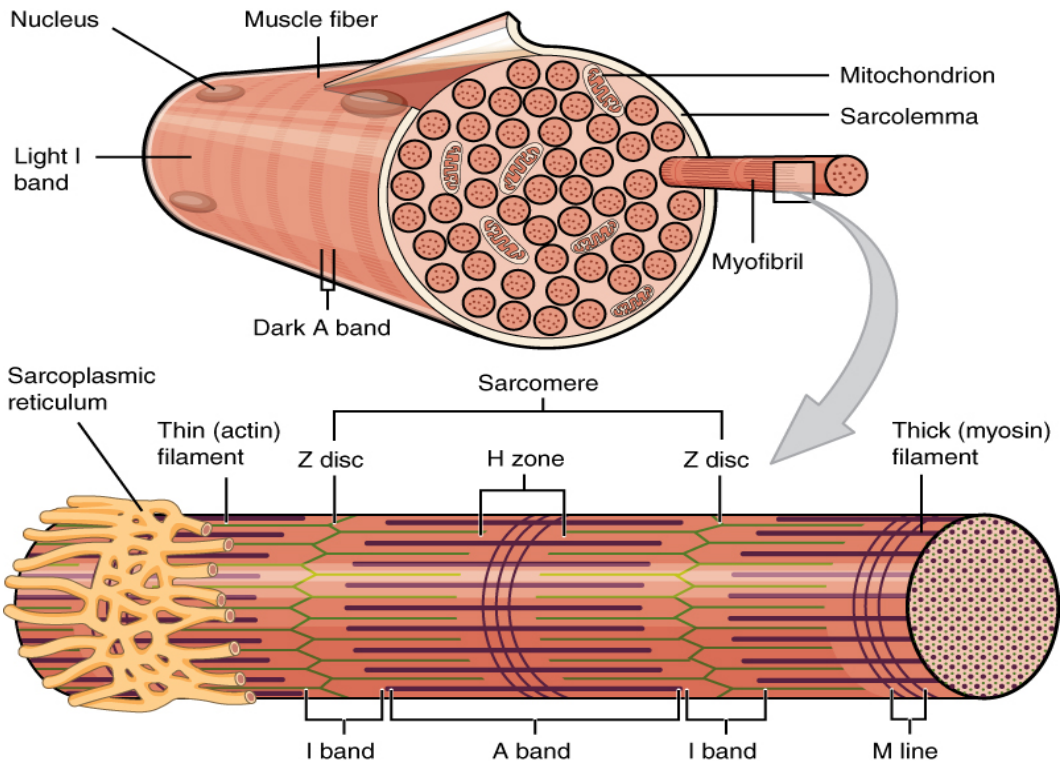

Figure 2. Structural organisation of individual muscle fibres.

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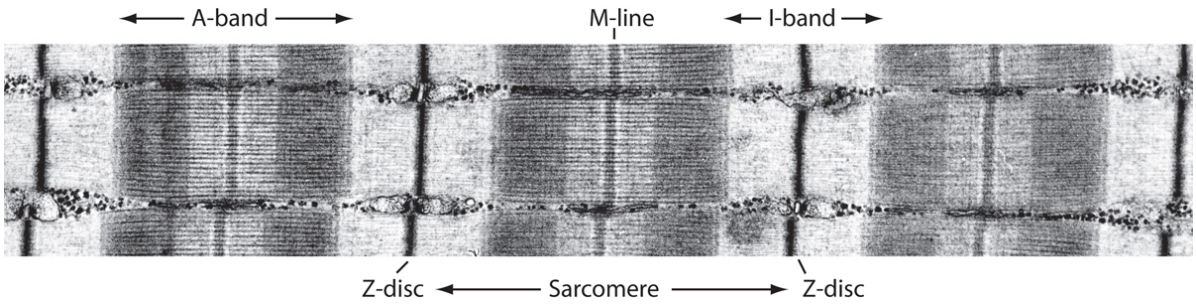

Figure 3. Electron microscopy image of a part of a myofibril showing sarcomeres with its bands and lines.

(From NIH Centre for Macromolecular Modelling & Bioinformatics; http://www.ks.uiuc.edu/~ericlee/Telethonin/; JPEG file: MuscleL1.jpg; accessed 13 March 2018)

Each myosin molecule contains two heavy chains (MyHC) and four light chains (MLC). The heavy chains constitute the tails, which aggregate to form the thick filaments, and globular heads, which project off to the sides and are able to bind to the myosin binding site situated on the adjacent actin molecules (Figure 4). Two types of light chains, regulatory (RLCs) and essential (ELCs, also known as alkali light chains) are associated with the globular heads of the MyHCs.[1,2] In the resting state, when the intracellular Ca^{2+} concentration is low (pCa^{2+} = 7.0), the myosin heads are prevented from interacting with actin by tropomyosin, which shields the myosin binding site on actin. The position of tropomyosin is in turn regulated by troponin, which in effect acts as a "lock" to secure tropomyosin in the shielding position.[3]

1.1.1.2 *The physiology of muscle fibre contraction*

During muscle contraction, the thin and thick filaments slide over each other, leading to shortening of individual sarcomeres.[4,5] This leads to shortening of the muscle fibres and, therefore, the muscle as a whole. The sliding of thin and thick filaments is caused by the interaction between myosin and actin, a process dependent on both ATP as a source of energy, and Ca^{2+} as a regulatory factor.

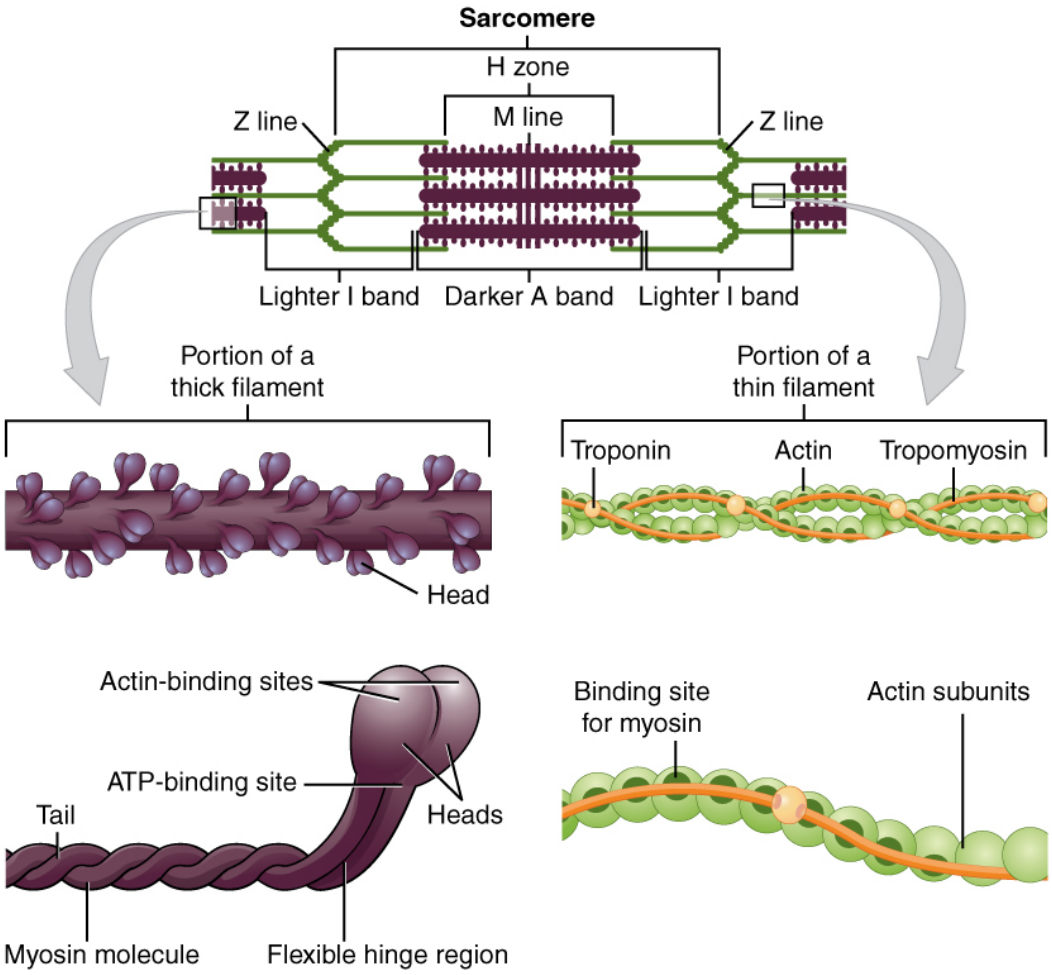

Figure 4. Ultrastructure of the sarcomere.

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As shown in figure 4, the myosin head contains binding sites for both ATP and actin. When ATP binds to myosin, chemical energy is released and ATP is hydrolysed into ADP and inorganic phosphate by the myosin ATPase enzyme – the latter forms part of the genetic encoding of the MyHC chain. The myosin molecule undergoes a conformational change with the head pivoted into an energised position (i.e. chemical energy is transformed into mechanical energy), analogous to the cocked hammer of a spring-loaded pistol. When actin binds with energised myosin at the actin binding site,

and on release of the bound P*i*, the stored mechanical energy in the myosin molecule is released and the myosin head pivots back to its resting state. This pivoting motion causes the myosin and actin filaments to slide over each other, leading to shortening of the sarcomere. However, each single pivoting motion of the myosin head results in a minute change in shortening of the sarcomere. Therefore, in order to produce the large movements required for contraction, the process needs to be repeated. This requires detachment of myosin from actin, a process that requires the binding of a new molecule of ATP to the myosin ATPase. The newly bound ATP can then be hydrolysed and the released chemical energy utilised to regenerate the energised form of myosin.

In order for contraction to occur in response to a neural stimulus (i.e. to regulate contraction), a process termed excitation-contraction coupling is required. The term "excitation-contraction coupling" refers to the consolidation of excitation of the sarcolemma to actual contraction of the muscle fibre. When the action potential spreads along the sarcolemma and T-tubules, membrane depolarization leads to an influx of Ca^{2+} ions via voltage-gated Ca^{2+} channels, in turn leading to opening of Ca^{2+} channels (ryanodine receptors) in the SR. These Ca^{2+}-induced Ca^{2+} release channels ensure the large and rapid supply of Ca^{2+} required for contraction.[3] The released Ca^{2+} ions attach to binding sites on troponin, leading to a structural change in troponin and a resultant altered interaction between troponin and tropomyosin in such a way that the myosin binding site on actin is exposed. When this occurs, the myosin-actin cross-bridges required for contraction can be formed. Muscle contraction ceases when Ca^{2+} is actively pumped back into the SR via sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps, returning the tropomyosin to its original form and shielding the myosin binding sites on actin.[3]

1.1.1.3 *Muscle fibre types*

Muscle fibres can be classified into different types based on the myosin ATPase activity at different pHs or antibodies directed at the various MyHC isoforms. Both histological classifications correlates well with the isoform protein concentration determined from biochemical techniques. Three main types can be distinguished, namely type 1 (slow twitch oxidative; MyHC-I), type 2A (fast twitch oxidative glycolytic; MyHC-IIa) and type 2B (fast twitch glycolytic; MyHC-IIx).6 Because of the MyHC composition, type 1, 2A and 2B are usually referred to as type I, IIA and IIX, respectively, and this nomenclature will be used in this thesis. Additionally, Smerdu et al. (1994) has shown that type IIB fibres have been misclassified as they contain transcripts of MHC IIx and not MHC IIb.7 By convention, upper case is used where immunohistochemical techniques are employed for typing (e.g. type IIA), and lower case where fibre types are identified by gel electrophoresis (e.g. type IIa). A fourth type, MyHC-IIb, is expressed in murine, but not human skeletal muscle.8 A fifth type (embryonic; MyHC-emb) can also be identified, mainly in regenerating fibres, as can be seen in muscle disorders such as muscular dystrophies.9 Fibres with combinations of MyHC isoforms (so-called hybrid fibres) can also be identified in biopsies.

Table 1 summarises the main composition, histochemical and mechanical properties of the different muscle fibre types.

	Muscle fibre type		
	I	IIA	IIX
MyHC composition	MyHC I	MyHC II	MyHC X
Histochemical properties			
Myosin ATPase staining intensity at pH 10.3	Low	High	High
Myosin ATPase staining intensity at pH 4.6	High	Low	Medium
Oxidative enzyme activity (SDH, CS, 3HAD, NADH, COX)	High	Medium	Low
Glycolytic capacity (LDH, PFK)	Low	Medium	High
Triglyceride content	High	Medium	Low
Glycogen content	Low	High	Medium
Mechanical properties			
Twitch contraction time (shortening velocity)	Slow	Fast	Fast
Maximum tetanic force	Small	Moderate	High
Fatigue resistance	Very high	Moderate/High	Low

Table 1. Features of the different muscle fibre types.

MyHC: myosin heavy chain; NADH: nicotinamide adenine dinucleotide, reduced; PAS: periodic acid Schiff; SDH: succinate dehydrogenase, CS: citrate synthase; 3HAD: 3-hydroxyacetyl co A dehydrogenase; LDH: lactate dehydrogenase; PFK: Phosphofructokinase. (Adapted from Karpati et al.[6])

1.1.1.4 *Normal muscle fibre type proportion and size*

In humans, reliable data for fibre type proportion and size are only available for the *Vastus lateralis* (VL) muscle. A meta-analysis of 18 studies estimated type I fibre proportion (± SD) in the VL to be 50.3 ± 1.9%, with no significant difference between men and women.[10] The same meta-analysis provided estimates for cross-sectional area (CSA) per fibre type in men and women; the means and pooled SDs per gender

and fibre type are provided in Table 2. It is interesting to note that this meta-analysis found type 1 fibres to be larger than type 2 fibres. However, a different paper reviewing data including 87 individuals (both men and women) from three different studies found the converse, type 1 and 2 fibre sizes being 4 754 ± 1 137 μm^2 and 5 145 ± 1 329 μm^2, respectively.[11] This underlines the fact that muscle fibre sizes appear to be extremely variable across different studies.

	Muscle fibre type		
	I	IIA	IIX
Men (n=141)	4 652 ± 1 391	4 167 ± 1 630	3 697 ± 1 577
Women (n=125)	3 840 ± 1 587	3 056 ± 1 598	2 033 ± 1 519

Table 2. Mean CSA (μm^2) ± SD for different muscle fibre types in men and women, reproduced from a meta-analysis by Gouzi et al. (2013).[10]

CSA: cross-sectional area; SD: standard deviation.

It is interesting to note the effect of immobilisation, on the one hand, and training on the other, on muscle fibre size. During periods of immobilisation, the size of both type I and II fibres decrease significantly.[12,13] Resistance training leads to an increase in size of both fibre types,[14,15] while pure endurance training may lead to a decrease in size of both fibre types.[16] However, despite the decrease in size, force remains unchanged or even increases in response to endurance training, resulting in an increase in specific force (SF; maximum force normalised to cross-sectional area). This underlines the fact that contractile force is not directly proportional to fibre size, and may have important implications for understanding adaptation of muscle fibres to other factors, such as disease.

1.1.2 *Muscle Disorders*

Muscle disorders (myopathies) in adults arise from a wide variety of hereditary and acquired aetiologies, and may be due to abnormalities in structural proteins (e.g. muscular dystrophies, critical illness myopathy), impaired metabolic pathways (e.g. disorders of carbohydrate, lipid or mitochondrial metabolism) or immune-mediated inflammation (e.g. idiopathic inflammatory myopathies). Although the aetiologies are heterogeneous, the unifying feature is that of muscle weakness that affects predominantly large proximal and axial muscle groups.

Theoretically, the force generated by a muscle is directly related to the number and firing frequency of recruited muscle fibres and the contractile force generated by each of the individual fibres.[17] Therefore, weakness, defined as a subjective (reported by an individual) or objective (found on clinical examination) impression of lack of strength, may result from either quantitative (decrease in the number of functional fibres) or qualitative (impaired contraction of individual fibres) factors (Figure 5). Disease processes that may lead to a decrease in the number of functional fibres include myofibre necrosis (due to inflammation, toxins etc.), impaired neuromuscular junction transmission (e.g. myasthenia gravis), and inexcitability of the sarcolemma (e.g. critical illness myopathy, ion channel disorders). Factors that may lead to impaired contractile function of individual fibres include structural abnormalities of proteins (e.g. muscular dystrophy), myofibrillary dysfunction, deficient cellular energy production (e.g. mitochondrial disorders, glycogen storage disorders etc.) and insufficient Ca^{2+} supply.

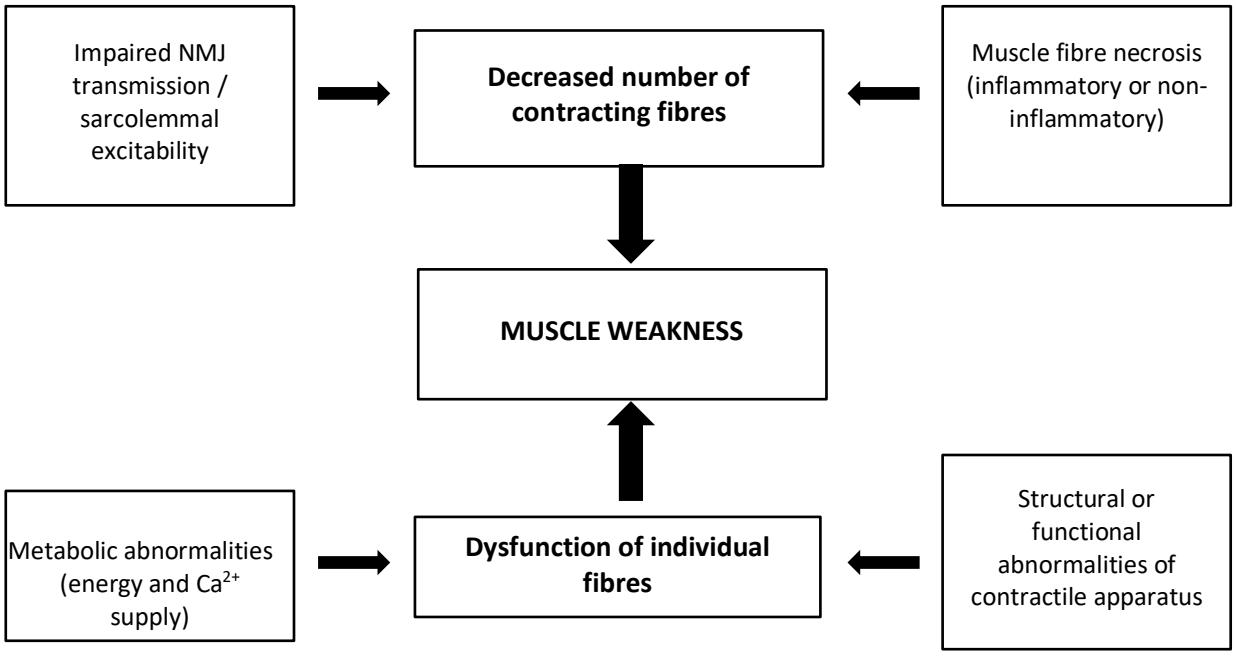

Figure 5. Potential pathogenic processes in muscles leading to muscle weakness.

Several hereditary myopathies are caused by mutations in genes coding for structural muscle proteins, e.g. dystrophinopathies, sarcoglycanopathies. Abnormal or dysfunctional structural proteins could theoretically lead to impaired contractility of muscle fibres, as has been shown to occur (although not conclusively) in some forms of muscular dystrophy.[18-20] As the disease progresses, loss of muscle fibres most likely contributes to the weakness, and is probably the reason for the progressive nature of these disorders.

In contrast to disorders affecting structural muscle proteins, the mechanism of weakness in acquired muscle disorders is less obvious. The most prevalent group of acquired muscle disorders is that of the idiopathic inflammatory myopathies (IIMs). The group of IIMs consists of polymyositis (PM), dermatomyositis (DM), inclusion body myositis (IBM), and necrotising autoimmune myopathy (NAM). Some authors also

include a group called non-specific inflammatory myopathy (NIM). Although IBM is also characterised by an inflammatory component, it differs from the other disorders in the group both from a clinical and pathogenic perspective. The distinguishing clinical characteristics include age of onset in the sixth decade or later, prominent finger flexor and pharyngeal weakness, slow onset and progression over years, delayed diagnosis (mean 5.2 years from onset of symptoms),[21] and only moderately raised serum CK,[22] while the pathogenesis is characterised by the presence of a prominent degenerative component.[23,24] IBM will, for these reasons, not be included in the remainder of the discussion.

IIMs are rare disorders, with an annual incidence of between 1.2 and 19 per million person-years.[25] These disorders affect individuals of all ages, and usually present as a subacute onset of symmetric proximal and axial weakness and fatigue (defined as a subjective sensation of fatigue and not an objective change in performance).[26-28] In the case of DM, characteristic skin involvement consisting of Gotron's papules, heliotrope rash, a shawl sign or a V-sign, may be present. On muscle biopsy, necrotic fibres, inflammatory cell infiltrates, major histocompatibility class I (MHC-I) antigen expression, and muscle fibre atrophy can be seen.[29] Table 3 summarises the currently accepted diagnostic criteria for the different entities comprising IIMs.

Although different entities are recognised within the group of IIMs, the classification of IIMs is not universally agreed upon.[30] Despite the fact that different pathological features and mechanisms are recognised for the respective entities, the similarities between them may perhaps overshadow the differences. These include (1) symmetrical proximal and axial weakness, (2) subacute course, (3) elevated serum muscle enzymes (creatine kinase) (4) inflammation, necrosis, and regeneration on

muscle histology, (5) myopathic features and “irritable” phenomena on electrophysiological examination (spontaneous activity on needle EMG examination indicating muscle fibre necrosis), and (6) responsiveness to immunosuppressive therapy. It could therefore be argued that, although accomplished through different pathogenic mechanisms, the unifying feature is that of weakness caused, in some way or another, by an inflammatory attack on muscle.

Of particular relevance to this research are the immunological processes at play in muscle in IIMs, as these processes are critical in explaining the resultant weakness and fatigue. In DM, C5b-9 membrane attack complex (MAC) is activated early and deposited on the endothelial cells. The activation of MAC leads to the release of proinflammatory cytokines and facilitates the migration of activated lymphocytes (B cells, CD4+ T cells and plasmacytoid dendritic cells), to the perimysial and endomysial regions. PM, by contrast, is due to a MHC-I-restricted, cytotoxic T cell-mediated destruction of muscle fibres, while an antibody-dependent complement-mediated lysis is most likely responsible in NAM.[26,31-33] However, despite differences in immunopathogenesis, a number of overlapping characteristics can be identified across all subtypes of IIMs. These include expression of MHC-I molecules on the surfaces of muscle fibres, as well as the presence of a number of pro-inflammatory cytokines, in particular interleukins (ILs), type I interferons (IFNs), and tumour necrosis factor-alpha (TNF-α).[34,35] The possibility that one or more of these cytokines could cause or contribute to weakness should be considered, and will be explored below.

Criterion	Dermatomyositis		Polymyositis	Non-specific inflammatory myopathy	Necrotising Autoimmune Myopathy
	Definite	Probable			
	All ages	All ages	>18 years	All ages	All ages
Myopathic muscle weakness	Yes*	Yes*	Yes*	Yes*	Yes*
Electromyo-graphic findings	Myopathic	Myopathic	Myopathic	Myopathic	Myopathic
Muscle enzymes in blood	High (up to 50 times normal)	High (up to 50 times normal)	High (up to 50 times normal)	High (up to 50 times normal)	High (up to 50 times normal)
Muscle biopsy findings	Perifascicular, perimysial or perivascular infiltrates; perifascicular atrophy.	Perifascicular, perimysial or perivascular infiltrates; perifascicular atrophy.	Primary inflammation, with the CD8/MHC-I complex and no vacuoles.	Non-specific inflammatory infiltrate without CD8/MHC-I complex. No perifascicular atrophy.	Ubiquitous MHC-I expression, but no/minimal inflammatory infiltrate.
Skin rash or calcinosis#	Present	Absent	Absent	Absent	Absent
*Myopathic muscle weakness, affecting proximal muscles more than distal ones and sparing eye and facial muscles, is characterised by a subacute onset (weeks to months) and rapid progression in patients who have no family history of neuromuscular disease, no exposure to myotoxic drugs or toxins, and no signs of biochemical muscle disease. #Calcium deposits in the skin					

Table 3. Diagnostic criteria for inflammatory myopathies. From Dalakas, Liang[26,36]

1.2 Synopsis of current literature

Most research on the pathogenic mechanisms of IIMs has focussed on the induction and maintenance of the inflammatory response, as well as the processes involved in immune-mediated muscle fibre necrosis. It is important to note that the focus on inflammatory necrosis of myofibres is based on the assumption that this phenomenon explains the weakness observed in these conditions. However, there is reason to believe that at least some of the observed muscle weakness is due to dysfunction of viable muscle fibres. The literature review will provide an overview of the potential mechanisms involved in the development of muscle weakness.

1.2.1 *Muscle fibre necrosis*

Weakness in IIMs is usually attributed to loss of muscle fibres due to inflammatory necrosis. However, there are a number of limitations that need to be considered before attributing weakness solely to loss of muscle fibres in these conditions. Firstly, there is poor correlation between the degree of inflammation and muscle weakness, and muscle weakness may in fact develop before the inflammatory infiltrate is present on muscle biopsy.[37] Secondly, the number of necrotic fibres observed on histological analysis is usually small. Very little quantitative data on fibre necrosis is available, and the presence of necrotic fibres is often described as “scattered”.[29,35,38] A single study quantified the presence of necrotic fibres in 13 patients with DM and 15 with PM, and found a mean of ~1% of fibres to be necrotic (range from 0-3%).[39] Thirdly, there is typically a clear-cut improvement in strength following treatment with corticosteroids, often becoming evident within 3-4 weeks.[35,38] It is unlikely that improvement in strength is due to restitution of

muscle fibre numbers, since muscle fibres are post-mitotic, and do not divide to form new fibres. An alternative explanation for the increase in strength due to corticosteroid treatment is hypertrophy of existing fibres. However, this argument is potentially negated by the fact that the histological hallmark of chronic treatment with corticosteroids is in fact type II fibre atrophy (with no or little change in type I fibres) and not hypertrophy.[40] Given the considerable difficulties that are raised when attributing muscle weakness to muscle fibre necrosis, it is highly likely that one or more different explanations exist.

1.2.2 *Structural or functional abnormalities of contractile apparatus*

Exogenous TNF-α has been shown to decrease contractile force in diaphragmatic and limb muscles in animal models.[41-44] Weakness develops within hours of administration (i.e. too early to be explained by muscle fibre atrophy and necrosis), and is induced by TNF-α levels that are too low to cause muscle atrophy. In addition, MHC-I upregulation appears to be related to reduced force-generating capacity in slow-twitch muscle of mice.[45]

A single study evaluating *in vitro* single muscle fibre function in untreated DM and IBM has been performed.[46] Five patients in each group were included, and compared with healthy controls. No differences in force generation between the two groups could be shown, and it was proposed that the force generating capacity of the remaining (surviving) muscle fibres are preserved in DM and IBM. However, this study had a number of shortcomings that could have influenced the results and will be discussed in Chapter 2. Therefore, muscle fibre contractility in IIMs remains largely unexplored.

1.2.3 *Metabolic abnormalities in muscle*

Although it is generally assumed that autoimmune and inflammatory processes are central to the muscle weakness in IIMs, a number of observations would suggest that non-immune mechanisms may contribute to the weakness. For example, there are dissociations between the degree of inflammation and weakness,[47,48] a subgroup of myositis patients do not respond to corticosteroids,[49] and in some patients, corticosteroid treatment leads to elimination of the inflammatory infiltrate from the muscle tissue with little or no improvement in strength.[50] One of the proposed metabolic defects in IIMs is an acquired deficiency of the enzyme AMP deaminase 1 (AMPD1, also known as myoadenylate deaminase), a rate-limiting enzyme in the purine cycle, and an important component of cellular energy production. In a mouse model of IIM, Coley et al. illustrated a significant reduction in AMPD1 mRNA, protein expression and enzyme activity.[37] Of further significance was the fact that the loss of enzyme activity and weakness appeared prior to the inflammatory infiltrate. These findings support the notion that weakness in IIMs may be due (at least partly) to metabolic factors and not only to inflammatory muscle damage. What is responsible for the deficiency in AMPD1 is not clear, but it is theoretically possible that cytokines may modulate AMPD1 expression in muscle. It has further been shown that IFNs and IL-1 are able to inhibit the expression of AMPD1 in vitro, and that IL-15 may have a stimulatory effect on the expression of AMPD1.[51] However, it should be noted that the clinical relevance of AMPD1 deficiency, whether inherited or acquired, is disputed, and current evidence suggests that AMPD1 deficiency is merely a coincidental finding.[52]

1.2.4 *Effect of corticosteroids on muscle*

Corticosteroids are the most effective anti-inflammatory drugs available for the treatment of chronic inflammatory conditions, including the IIMs, and examining the effects of this medication on the inflammatory response in muscle tissue may provide valuable indirect insights into the pathogenic mechanisms. Down-regulation of inflammatory gene expression encoding multiple inflammatory proteins (e.g. cytokines, chemokines etc.) is the most important anti-inflammatory effect of this group of drugs.[53] In muscle biopsies of IIM patients treated with corticosteroids, there is decreased expression of particular cytokines, including IL-1α and IL-1β.[50] As noted above, IL-1 may inhibit the expression of AMPD1, and corticosteroids may therefore indirectly increase the expression of AMPD1 through this mechanism. Corticosteroids also inhibit the secretion of TNF by monocytes,[54] and also decrease serum levels of soluble TNF-α receptors (TNFR1 and TNFR2) in other autoimmune diseases.[55]

However, corticosteroids may have other non-immune effects on muscle. The effectiveness of corticosteroids in the treatment of Duchenne muscular dystrophy, an inherited muscle disorder due to mutations in the gene coding for dystrophin, illustrates this well. A number of hypotheses about the mechanism of action in this condition have been put forward and include a positive effect on myogenesis,[56,57] an anabolic effect on muscle (resulting in increased muscle mass),[58] and an increase in resting intracellular Ca^{2+} concentration.[59] Whether any of these mechanisms are important in IIMs remains to be explored.

1.2.5 *Summary of literature*

In addition to inflammatory necrosis of muscle fibres, other effects on muscle, both inflammatory and non-inflammatory, may contribute significantly to weakness in IIMs. Contractility of muscle fibres in IIMs has not adequately been explored, although a number of observations would suggest that dysfunction of the contractile apparatus might contribute to muscle weakness in these conditions. These include an acquired deficiency of AMPD1, depression of muscle fibre contractility by TNF-α, a rapid response to treatment with corticosteroids, and dissociation between the degree of inflammation and weakness.

It therefore appears unlikely that a decrease in the number of muscle fibres due to inflammatory necrosis is the sole mechanism responsible for weakness in IIMs, and it is probable that qualitative changes in muscle fibre contractility play an important role. These factors have not been adequately explored, and warrant careful investigation.

1.3 Aims and objectives

Central to an investigation into the possible mechanisms involved in the development of weakness in IIMs, is a better understanding of the effect of inflammatory muscle disease on the functional integrity of the contractile apparatus of the muscle fibre. This research project set out to achieve this by means of the following objectives:

i. Morphological and metabolic assessment of muscle tissue from patients with IIMs. Morphological assessment included quantification of the presence of necrotic muscle fibres, (2) fibre size and (3) fibre type, while metabolic function was investigated by means of enzyme analysis.

ii. Assessment of the maximum absolute and normalized (specific) force generated by single muscle fibres from patients with untreated IIMs compared to healthy volunteers.
iii. Assessment of the maximum velocity and power generation ability of single muscle fibres from patients with untreated IIMs compared to healthy volunteers.
iv. Assessment of the Ca^{2+} sensitivity of single muscle fibres from patients with untreated IIMs compared to healthy volunteers.

1.4 Hypothesis

The hypothesis is that weakness in IIMs is related not only to a decrease in the number of muscle fibres (i.e. a structural abnormality due to inflammatory necrosis), but also to abnormalities of the contractile properties of the individual muscle fibres (i.e. a functional abnormality). These abnormalities may manifest as one or more of the following:

i. Impaired generation of maximum SF of individual muscle fibres.
ii. Alterations of contractile velocity and power output of individual muscle fibres. In the face of impaired force generation, it is expected that there would be a compensatory increase in maximum shortening velocity of muscle fibres in order to maintain power output.
iii. Increased Ca^{2+} sensitivity of muscle fibres, in order to compensate for impaired force generation.

1.5 Conclusion

In addition to inflammatory necrosis of muscle fibres, other effects on muscle, both inflammatory and non-inflammatory, may contribute significantly to weakness in IIMs. Contractility of muscle fibres in IIMs has not adequately been explored, although a number of observations would suggest that dysfunction of the contractile apparatus might contribute to muscle weakness in these conditions. These include an acquired deficiency of AMPD1, depression of muscle fibre contractility by TNF-α, a rapid response to treatment with corticosteroids, and dissociation between the degree of inflammation and weakness.

It therefore appears unlikely that a decrease in the number of muscle fibres due to inflammatory necrosis is the sole mechanism responsible for weakness in IIMs, and it is probable that qualitative changes in muscle fibre contractility play an important role. These factors have not been adequately explored, and warrant careful investigation. It is important to note that the study was designed to investigate whether the contractility of structurally intact (i.e. non-necrotic) muscle fibres is affected in patients with IIMs, and not to determine what the reason for impaired contractility (if present) is. This could form the basis for future research, and will be discussed in the final chapter of the thesis.

CHAPTER TWO

2 QUANTITATIVE ANALYSIS OF MORPHOLOGIC AND METABOLIC CHARACTERISTICS OF MUSCLE BIOPSIES FROM PATIENTS WITH IDIOPATHIC INFLAMMATORY MYOPATHIES

2.1 Introduction and synopsis of current literature

The diagnosis and classification of the IIMs is heavily dependent on the morphological features displayed on muscle histology, and the qualitative histological features of the IIMs have been extensively examined and documented. In DM, these features include a perivascular inflammatory infiltrate consisting of predominantly CD4+ T helper cells, perifascicular atrophy, major histocompatibility complex class I (MHC-I) antigen expression in the perifascicular regions, and the presence of scattered necrotic and regenerating muscle fibres.[29,60] In PM, the typical findings include an endomysial and perimysial inflammatory infiltrate consisting of predominantly cytotoxic CD8+ T cells, widespread MHC-I antigen expression, the invasion of non-necrotic muscle fibres by CD8+ lymphocytes, and scattered necrotic and regenerating muscle fibres.[29,60] NAM is characterised by randomly distributed necrotic muscle fibres and regenerating fibres, little or no inflammatory infiltrate, and weak or no MHC-I expression.[29,60] In contrast, there is a paucity of quantitative histological data in IIMs. For example, the number of necrotic fibres on muscle biopsy specimens from patients with DM and PM has only been

documented in a single study.[39] Very little quantitative data on muscle fibre size or fibre type proportion is available. A single paper described mean fibre diameters in IIMs, but a detailed description of the methodology (in particular whether smallest or largest diameters were measured) was not provided, and comparison to a healthy control group was not made.[61] In both clinical practice and research, reporting of these parameters is generally limited to subjective descriptions. Although such an approach is unlikely to affect the overall validity of the histological interpretation, it could be argued that quantitative data may provide more useful information on the underlying pathogenic mechanism of these disorders. For example, if fibre type proportion is found to be altered in IIMs, this may indicate an increased vulnerability to inflammatory necrosis or impaired regenerative capacity of one fibre type.

In addition to morphological features of the IIMs, data on the metabolic function of muscle fibres may provide useful insights into the capacity of affected muscles to generate and maintain force production. A few studies have investigated mitochondrial function in DM and /or PM by means of enzyme histochemistry, biochemical assays, or both.[62-65] Enzyme histochemistry, utilising cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) reactions, revealed features suggestive of electron transport chain (ETC) abnormalities in most cases, but results of biochemical analysis were less consistent, with not all studies confirming ETC abnormalities. Proteomic analysis was performed in one of these studies, and revealed down-regulation of electron transport chain (ETC) subunits, assembly factors, and tricarboxylic acid (TCA) cycle enzymes.[63] Another study evaluated citrate synthase (CS), a mitochondrial matrix enzyme involved in the Krebs cycle, and 3-hydroxyacyl-CoA dehydrogenase (3-HAD), an enzyme involved in

mitochondrial beta-oxidation, in patients with PM and DM before and after a 12-week endurance training program.[66] The investigators found a significant increase in enzyme activity after the training program.

2.2 Purpose of the study

2.2.1 *Aims and objectives*

The aims of the study were to determine whether there are morphological and/or biochemical abnormalities present in muscle of patients with IIMs that could (at least in part) explain the weakness and fatigue that patients with these disorders experience. In order to achieve this, the objectives of the study were:

i. To determine the mean CSA of each muscle fibre type in muscle from patients with IIMs and compare it to healthy controls.
ii. To determine the fibre type proportion in muscle from patients with IIMs and compare it to healthy controls.
iii. To measure the activities of enzymes involved in different metabolic pathways in muscle from patients and compare it to healthy controls.
iv. To quantify the amount of necrosis present in muscle from patients with IIMs

2.2.2 *Hypotheses*

It is hypothesized that the mean CSA of all fibre types is decreased in IIMs, but that fibre type proportion is unaffected. Furthermore, based on previous data suggesting abnormalities in the electron transport chain, as well as the reported early fatigue in

patients with IIMs, it is hypothesized that enzyme analyses would reveal the presence of metabolic abnormalities. In addition, based on limited data,[39] it is hypothesized that quantitative analysis will reveal a relatively low number of necrotic fibres in muscle biopsies from IIM cases.

2.3 Methods

2.3.1 *Sources of muscle biopsy specimens*

2.3.1.1 *Cases*

Participants were recruited from the in- and outpatient departments of the Divisions of Neurology and General Medicine, Tygerberg Hospital. Only patients who were to undergo diagnostic muscle biopsies were asked to participate. All adult patients in Tygerberg Hospital possibly requiring diagnostic muscle biopsies are referred to the Division of Neurology for assessment, and all diagnostic muscle biopsies are performed in the Division of Neurology by or under supervision of the principal investigator. Following informed consent, the muscle specimens for study purposes were obtained during the biopsy done for diagnostic purposes, and no biopsies were done for the sole purpose of this study. Modified Rankin Scale (MRS) assessment (Table 4), as a marker of disability, was done at the time of the muscle biopsy.

Patients meeting the following criteria were included:

i. Age 18 years or older.
ii. A diagnosis of PM, DM, or NAM.

Note: because a diagnostic muscle biopsy is necessary for confirming the diagnosis, definitive diagnoses were not available at the time of the biopsy. Therefore,

muscle tissue was obtained from all patients with a likely diagnosis of one of above, but only tissue from participants in whom the diagnosis has been confirmed were further assessed (immunohistochemistry, enzyme analysis and contractility studies were postponed until a definitive diagnosis had been made). The final diagnoses were based on accepted clinical, laboratory, electromyographic and histological criteria (as discussed in Chapter 1) and a response to treatment with corticosteroids at 6 weeks after treatment initiation. IBM cases were excluded from the study, as IBM differs significantly from the other IIMs in terms of pathogenesis (prominent degenerative component), course (slowly progressive), time to diagnosis (mean 5.2 years), and response to treatment.[21,23,24]

Patients with any of the following were excluded:

i. Recent treatment with intravenous immunoglobulin, plasmapheresis or corticosteroids (within the previous 6 weeks before the biopsy) or steroid-sparing agents (within the previous 6 months before the biopsy).
ii. Treatment with oral anticoagulants (e.g. warfarin), as this is a contra-indication to performing a muscle biopsy.
iii. The presence of any other neurological or muscle disorder, as these may potentially influence the results of the contractility studies and biochemical analyses.

Score	Description
0	No symptoms at all
1	No significant disability despite symptoms; able to carry out all usual duties and activities
2	Slight disability; unable to carry out all previous activities, but able to look after own affairs without assistance
3	Moderate disability; requiring some help, but able to walk without assistance
4	Moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance
5	Severe disability; bedridden, incontinent and requiring constant nursing care and attention
6	Dead

Table 4. The Modified Rankin Scale.

2.3.1.2 *Controls*

Controls were healthy adults (older than 18 years), male or female, and from all ethnic groups, who were volunteers participating in approved studies within the UCT/MRC Research Unit for Exercise Science and Sports Medicine (ESSM). Their muscle samples served as a comparison for the diseased muscle for enzyme analysis. These individuals were informed about this additional research study and assured that the donation of a small part of their biopsy would not affect the overall outcome of the study in which they were currently participating. A separate information sheet and consent form were provided for this purpose. We attempted to match healthy volunteers to cases for both age and sex as closely as possible. For comparison of morphometric data (fibre type

proportion and CSA), we utilized published reference values (see section on Statistical analysis).

2.3.2 ***Specimen acquisition and storage***

2.3.2.1 *Cases*

The muscle samples for study purposes were obtained from the muscle tissue acquired for diagnostic purposes. Biopsies were performed by the investigator under local anaesthesia using standard techniques for an open biopsy. Measurement of the contractile properties of single muscle fibres requires only a small sample of tissue, which can be obtained by needle biopsy. However, to ensure the acquisition of sufficient tissue for diagnostic purposes, open biopsies and not needle biopsies are preferred and is standard practice in most centres worldwide. All biopsies were taken from the *vastus lateralis* muscle via an incision 2-3 centimetres anterior to the midpoint of a line connecting the greater trochanter and the superior margin of the patella.

Fresh muscle specimens were divided into 2-3 samples of approximately 6mm x 4mm x 4mm, rapidly frozen in liquid nitrogen and stored at -200°C until analysis.

2.3.2.2 *Controls*

Muscle samples were obtained from the *vastus lateralis* of participants by means of a Bergström needle biopsy under local anaesthesia. Access to the muscle was gained via an incision at the same site as those used for open biopsy in controls (2-3 centimetres anterior to the midpoint of a line connecting the greater trochanter and the superior margin

of the patella). One to three samples of approximately 4mm x 4mm x 4mm were obtained, rapidly frozen in liquid nitrogen and stored at -200°C until analysis.

2.3.3 ***Preparation and analysis***

2.3.3.1 *Morphometric analyses (fibre typing and CSA) of muscle from IIM cases*

Fibre type and CSA were determined by means of fluorometric immunohistochemistry. Serial sections of 10 µm thickness were cut at -22°C using a Leica CM1100 cryostat. The sections were mounted on glass slides and allowed to dry at room temperature for one hour, after which they were stored at -20°C overnight until further processing. Slides were blocked with 5% bovine serum albumin (BSA) for 1 hour in a humidifying box at room temperature, and then incubated overnight at 4°C with primary antibodies against MyHC I (BA-D5), MyHC IIA (SC71) and MyHC IIX (6H1), all three at a concentration of 1:50. These antibodies were obtained form Developmental Studies Hybrydoma Bank (DSHB, Iowa, USA). Following incubation, slides were washed twice with 0.15M phosphate buffered saline (PBS) and then incubated for 2 hours at room temperature with fluorescent-tagged secondary antibodies (AMCA goat anti-mouse IgG2b and AlexaFluor 488 goat anti-mouse IgG1; Jackson Immunoresearch Laboratories, Pennsylvania, USA). After incubation, slides were washed and mounted with fluorescent mounting media (Mowiol), and left overnight in the dark at room temperature. Slides were viewed with a Nikon Eclipse i80 fluorescent microscope and images were captured for analysis at 100x magnification with a Canon EOS 650D digital camera.

During image analysis, fibres were classified as type I, IIA, IIX, I/IIA hybrid, I/IIX hybrid or IIA/IIX hybrid (Figure 6) based on colour differentiation according to the fluorescent-tagged secondary antibody, and the fibre type proportion was calculated for each of the IIM specimens. After typing, the circumference (C), expressed in μm, of each fibre was measured and CSA, expressed as μm^2, calculated using ImageJ pre-calibrated software (NIH, Bethesda, Maryland, USA). All fibres in each captured image were included in fibre type proportion calculation, but only type I, type IIA and type IIX fibres were included in CSA analysis, due to the paucity of comparative data in the literature. For CSA analysis, all fibres of each type (I, IIA and IIX) in each biopsy, up to a maximum of 40 fibres per type per biopsy, were measured. Care was taken to measure only cross-sectional and not longitudinally oriented fibres. Mean CSA was calculated per fibre type for all biopsies combined.

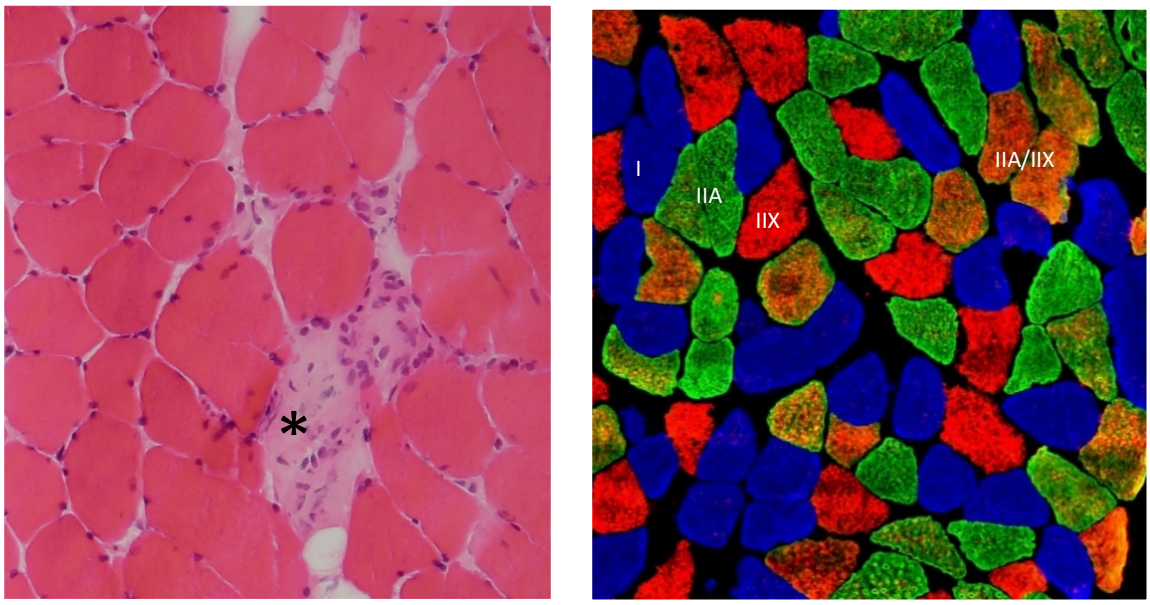

Figure 6. (A) H&E stained frozen section of muscle from a patient with necrotising autoimmune myopathy (NAM) showing myofibre necrosis (asterisk). (B) Example of images obtained by fluorometric immunohistochemistry of 10 µm thickness sections of muscle.

For illustrative purposes, only parts of the images are shown. The images illustrate type I fibres (blue), type IIA fibres (green) and type IIX fibres (red). Orange or green/red combined fibres represent type IIA/IIX hybrid fibres, while purple or blue/red combined fibres (not shown) represent type I/IIX hybrid fibres.

2.3.3.2 *Enzyme activities*

Lactate dehydrogenase (LDH), 3-hydroxyacyl-CoA dehydrogenase (3-HAD), citrate synthase (CS) and phosphofructokinase (PFK) maximal activities were determined using the fluorometric methods described by Essen-Gustavsson et al. (1984) and Kohn et al. (2007).[67,68] First, a muscle sample from each participant (each with a wet weight of ± 40 mg) was freeze-dried over night at -50°C under vacuum. 0.1 M potassium phosphate homogenising buffer (pH 7.3) was then added to a small portion of the freeze-dried muscle sample at a ratio of 400 µl buffer for every 1 mg of tissue, after which the sample

was pulse-sonicated on ice for three sessions of ten seconds each. The total protein concentration of each homogenate was determined using the Bradford protein assay.[69] A BSA stock solution was used to produce a series of standard BSA solutions with concentrations ranging from 0.0 – 0.5 g/l, which were pipetted into wells of a 96-well microplate and Bradford reagent added. A volume of 10 µl of each muscle homogenate was then pipetted in duplicate into separate wells of the microplate and 250 µl Bradford reagent added to each well. After incubation for 5 minutes at room temperature, the spectrometric absorbance was measured at 595 nm. A standard protein curve was constructed from the known BSA concentrations and fitted with a linear equation, which was utilised to calculate the protein concentration of each muscle homogenate.

Next, an NADH standard curve was generated by measuring the fluorescence of different known concentrations of NADH (excitation wavelength 340 nm; emission wavelength 460 nm), and the slope determined (fluorescence/µM NADH). Finally, enzyme activities in the muscle homogenates were determined by recording the emission at 460 nm for 5 minutes with 30-second intervals, using an excitation wavelength of 340 nm. A volume of 250 µl of the enzyme reagent was used for all assays; sample volumes were 3 µl for the LDH assay and 5 µl for the 3-HAD, CS and PFK assays. All enzyme activities were expressed as µmol/min/g protein.

2.3.3.3 *Quantification of muscle fibre necrosis*

For quantification of muscle fibre necrosis, either haematoxylin and eosin (H & E) or Gomori trichrome stained frozen sections (1 per IIM case) were analysed. Each slide was photographed, and all fibres in each captured image were counted. Thereafter, the

number of necrotic fibres in each image was determined, and confirmed by a qualified neuropathologist (Dr D Zaharie, EFN). The percentage of necrotic fibres for each IIM case, as well as the mean percentage of necrotic fibres, was subsequently calculated.

2.3.3.4 *Statistical analyses*

For comparison of morphometric data, we used published reference values from a meta-analysis rather than our own control data. Since published values on muscle fibre size appears to be highly variable (see Chapter 1), data from a large meta-analysis would provide better reference values than a limited number of controls from a single centre. For analysis of enzyme activities, we compared enzyme activities in muscle homogenates from IIM cases and controls.

Statistical analysis was performed using Graphpad Prism version 7 (GraphPad Software, La Jolla California USA).). For all parameters, mean ± SD was calculated, unless stated otherwise. Variables were compared using the Mann-Whitney *U* test for non-parametric data. Statistical significance was set at $P < 0.05$.

2.4 Results

Participants in the study consisted of six healthy controls (two male, four female) and five patients with IIMs (all female). The IIM group consisted of one patient with PM, two with DM, and two with NAM. The participant details are summarised in Table 5.

	GROUP	
	IIMs	**CONTROLS**
Age	48 (26-60)	30 (22-44)
Gender	5F	2M, 4F
Diagnosis	1 PM, 2 DM, 2 NAM	Healthy, recreationally active
Modified Rankin Scale	3 (1-5)	0

Table 5. Participant details. **Age is reported as mean (range) in years. Modified Rankin Scale is reported as median (range). By definition, the MRS of all controls were 0 (no symptoms).**

DM: dermatomyositis; PM: polymyositis; NAM: necrotising autoimmune myopathy; F: female; M: male.

2.4.1 *Fibre type proportion and CSA*

Morphometric analysis of IIM specimens revealed a mean type I fibre proportion of 36 ± 16%. This was substantially lower than published reference values for women (mean 48.1 ± 2.6%),[10] as well as previously published summary data from 90 controls (men and women; 87 historical).[11] However, the mean type I proportion ranged from 18 – 61% in the 5 IIM cases, suggesting significant differences within the group (Figure 7). This observation is further supported by the relatively large SD, indicating a wide distribution around the mean.

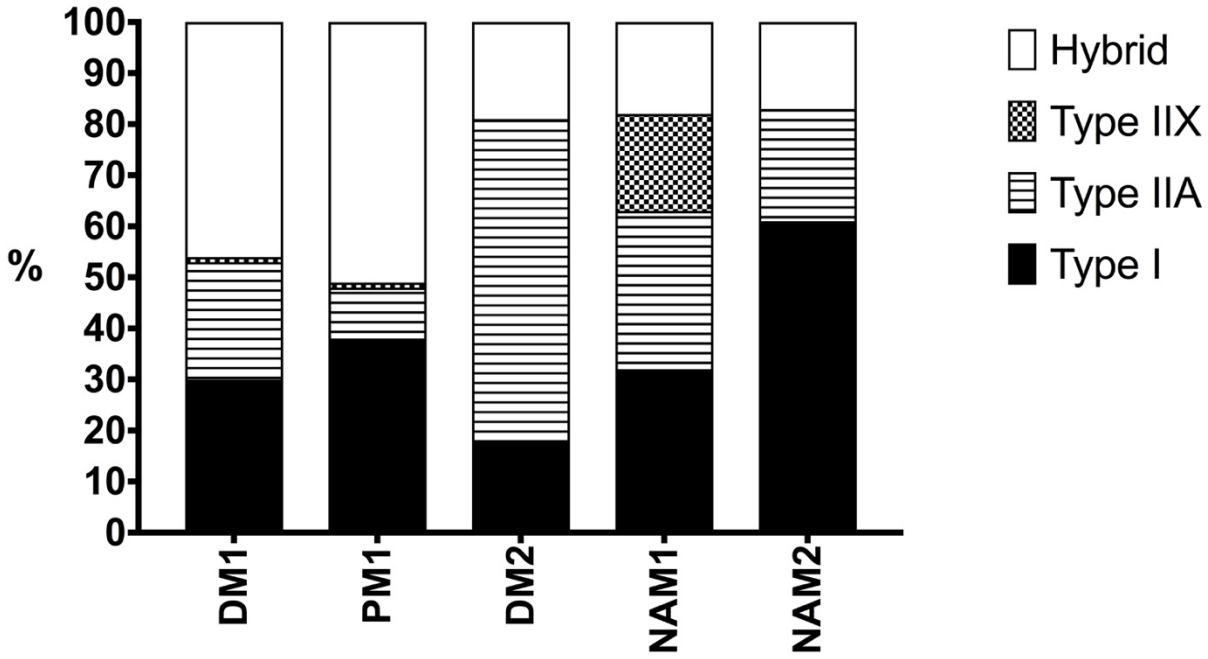

Figure 7. Fibre type proportions (%) for each IIM case.

DM: Dermatomyositis; NAM: Necrotising autoimmune myopathy; PM: Polymyositis.

CSA analysis did not reveal a decrease in fibre size in IIM cases (Table 6). In fact, mean CSA was higher than published reference values for women for all three fibre types,[10] but similar to other published data that included predominantly men.[11] Only the difference in type IIX CSA (compared to reference values) reached statistical significance ($p=0.03$).

Cross-sectional area (μm^2)			
	I	**IIA**	**IIX**
IIM cases	5 397 ± 2 779	5750 ± 3566	6726 ± 3163
All type II combined		5 621 ± 3 290	
Published reference values: women (Gouzi 2013)[10]	3 840 ± 1 587	3 056 ± 1 598	2 033 ± 1 519*
Published data: men and women (Henning, 2017)[11]	4 754 ± 1 137	5,145 ± 1,329	

Table 6. Cross-sectional area (CSA) for the vastus lateralis muscle in IIM cases and healthy controls.

Values provided are means ± SD. The study by Henning et al. (2017) included data from 87 historical controls and 3 current controls, of which 89 were men.

**Statistically significant difference compared to IIM cases from present study.*

2.4.2 *Enzyme activities*

Results of fluorometric enzyme activity analysis are shown in Table 7. One IIM sample was omitted from PFK analysis because of a missing value. Although the activities of all measured enzymes were decreased in IIM cases compared too controls, the difference reached statistical significance for only two of the enzymes (3-HAD and CS).

	IIMs	CONTROLS	p-value
Lactate dehydrogenase (LDH)	365.00 ± 84.74	484.60 ± 262.80	0.79
3-hydroxyacyl-CoA dehydrogenase (3-HAD)	33.60 ± 10.83	66.82 ± 38.5	0.03
Citrate synthase (CS)	13.06 ± 6.35	35.94 ± 15.69	<0.01
Phosphofructokinase (PFK)	144.2 ± 84.58	199 ± 64.96	0.15

Table 7. Fluorometrically measured enzyme activities in muscle homogenates of IIM cases and healthy controls.

Enzyme activities are means ± SD and expressed as µmol/min/g protein.

2.4.3 *Muscle fibre necrosis*

A total number of 2833 fibres were counted, with a mean of 567 fibres per IIM case (range 451 – 691). Of these, 30 fibres were found to be necrotic (1.1% of all fibres). The mean (± SD) percentage of necrotic fibres per participant was 1.1 ± 0.36%, with a range of 0.5 – 1.5%.

2.5 Discussion

The aim of this study was to investigate whether muscle fibre type distribution and size, as well as metabolic pathways, are affected in IIM. In addition, the magnitude of inflammatory necrosis in muscle from patients with these conditions was quantified.

At 36%, the mean proportion of type I fibres in IIM muscle was lower than previously published data and reference values for healthy subjects, and 3 of the 5 IIM cases had mean type I proportions below 32.9%, the lower limit of normal (LLN) for healthy subjects.[10] Two previous studies investigated fibre type proportion in the *vastus lateralis*

muscle of patients with IIMs. In an initial study by Dastmalchi et al. (2007) fibre type proportion was analysed in 9 patients with IIMs (5 PM, 4 DM) before and after a 12-week training program.[70] They found a type I proportion of 32 ± 10% in IIM cases, versus 47 ± 16% in controls. However, they studied only chronic, stable IIM cases, with a median disease duration of 22 months. All patients had undergone at least 1 year of immunosuppressive treatment, consisting of prednisolone with or without a steroid-sparing agent. Therefore, whether the low type I proportion represented disease pathology or a treatment effect is not clear, as corticosteroid treatment is associated with a decrease in type I fibre proportion.[71] Interestingly, the proportion of type I fibres increased to 42 ± 13% after training. In a further study by Loell et al. (2011) muscle biopsies from 38 patients with PM or DM were assessed , including 18 newly diagnosed, untreated patients, and 20 patients with chronic disease (more than 12 months disease duration post-diagnosis).[72] The investigators found a decrease in type I fibre proportion in chronic disease (32 ± 16% in non-responders and 36 ± 10% in responders), but not in untreated patients (48 ± 14%) or healthy controls (47 ± 16%). They concluded that the low type I proportion was not a characteristic of PM or DM per se, but observed in treated patients with chronic disease. The findings from the present study therefore do not conform to those from the latter study, and warrant further exploration. In theory, a lower type I (and hence an increase in type II) fibre proportion could be a compensatory response, as peak force and power production by type II fibres is generally higher.[73-75]

Unexpectedly, mean CSA of all three fibre types (I, IIA, IIX) were found to be increased in IIM cases, compared to published data and reference values. Although the difference reached statistical significance only for type IIX fibres, this is almost certainly

due to the small number of IIM cases included. Although variations in muscle fibre size and muscle fibre atrophy (and not hypertrophy) have been reported since the 1960's,[76,77] data using standardized methods for quantification is sparse. Loell et al. (2011) assessed CSA of type I and type II fibres in 18 untreated and 20 treated PM and DM cases, and compared it to healthy controls.[72] No differences were found between any of the groups. It therefore appears that results of muscle fibre size analysis should be interpreted with caution, as findings are heterogeneous across studies, including data from healthy controls. In their meta-analysis of data from 19 studies, including 423 healthy subjects, Gouzi et al. (2013) found significant heterogeneity between included studies, with an I^2 index of 36%.[10] Mean fibre CSA ranged from 2 858 ± 648 μm^2 to 5 892 ± 1095 μm^2 between studies. Based on these findings, it is prudent to avoid over-interpretation of CSA values, in both health and disease.

Enzyme analysis revealed significantly decreased activities of both 3-HAD and CS in IIM muscle samples, indicating impaired fat and carbohydrate oxidative capacity. Although LDH and PFK activities were also decreased, the differences did not reach statistical significance, and the effect sizes were also smaller. Whether lactate metabolism and glycolysis are also affected therefore remains unclear. A previous study investigating the effect of a 12-week endurance training program on exercise capacity in patients with stable IIMs was able to show both clinical improvement and increased muscle mitochondrial enzyme activities (CS and 3-HAD).[66] However, enzyme activity was not measured in healthy controls for comparison at baseline or after training, and it is therefore not clear whether the increase in enzyme activities happened against a background of impaired activity at baseline. It is well established that endurance training

leads to increased activity of muscle mitochondrial enzyme activities, both in healthy humans and in rats.[78-82] It is therefore reasonable to expect a similar response to endurance training in diseased muscle, although the magnitude may differ. Whether the metabolic abnormalities illustrated in the present study contribute to the development of weakness in IIMs or are simply the result of the disease process, therefore remains to be investigated. Furthermore, since enzyme activity appears to correlate with physical activity, the decreased activities shown in the present study may merely be a marker of physical inactivity due to muscle weakness. This hypothesis is supported by the fact that the median MRS of IIM cases was 3 (moderate disability; requiring some help, but able to walk without assistance), indicating significant limitation on activity.

In keeping with a previous study,[39] quantitative analysis showed that ~1% of fibres in muscle biopsies from IIM cases are necrotic. This relatively low number suggests that widespread necrosis is unlikely to be the main contributor to weakness observed in this condition. It could be argued that muscle fibre necrosis is an ongoing process leading to cumulative loss of muscle fibres, while muscle histology provides only a snapshot of the process at a particular time. Although this may potentially explain weakness in longstanding or treatment-resistant cases, it would not explain recent onset cases with clear weakness despite minimal loss of fibres on histology.

Study limitations

The main limitation of the study is the relatively small number of muscle biopsies from IIM cases that were analysed, which is related to the rarity of these disorders. Whether the findings are representative of this group of disorders is therefore uncertain.

However, many of the findings were in keeping with previously published data. Of particular interest are the unexpected findings of increased fibre size in IIM. Again, because of the small number of biopsies, reliable comparison of muscle fibre size between patients with IIMs and healthy controls could not be made, because of the small number of IIM cases included in the study. Another limitation is incomplete matching for age and gender. Because we utilized opportunistic recruitment and sampling, it was not possible to fully match cases and controls. However, this could only potentially have an influence on the enzyme analysis, as muscle fibre type and size was compared to the published literature and not healthy controls.

2.6 Conclusion

The main findings of the study are that, in addition to the well-known histological features, morphometric and metabolic alterations are present in muscle of patients with IIMs. Although muscle fibre necrosis is a common and characteristic feature of IIMs, its contribution to symptomatology, notably weakness, remains unproven and can probably be regarded as negligible based on the finding of this and other studies. It is a distinct possibility that these morphometric and metabolic changes are merely consequences of the disease process and not instrumental in the development of weakness.

CHAPTER THREE

3 *IN VITRO* CONTRACTILE FORCE MEASUREMENT OF SINGLE MUSCLE FIBRES FROM PATIENTS WITH IDIOPATHIC INFLAMMATORY MYOPATHIES

3.1 Introduction and background

Force generated by a muscle is directly related to the number of contracting muscle fibres and the contractile force generated by each of the individual fibres. Therefore, weakness of a muscle may result from either quantitative (decrease in the number or size of functioning muscle fibres in the muscle) or qualitative (impaired function of contractile apparatus of individual muscle fibres) factors.

Multiple processes may lead to a decrease in fibre number or size, such as myofibre necrosis, ischemia and denervation. However, the primary outcome from these processes is a decrease in the total number of actin-myosin cross-bridges that participate in force generation. In addition, contractile apparatus function (which is dependent on the interaction between actin and myosin filaments) may be affected by numerous factors, including abnormalities in structural proteins, impaired energy supply, alterations in calcium homeostasis, or combinations thereof.

The assessment of muscle strength can be performed on multiple levels, including:

- System level: Timed functional tests, e.g. rising from a chair, 30 metre walk test.

- Organ/muscle level: Manual muscle testing, dynamometry.
- Cellular/muscle fibre level: *in vitro* permeabilised single muscle fibre contractility testing.
- Molecular level: *in vitro* motility assay to measure the translational velocity of actin filaments on myosin.

In vitro single muscle fibre contractility studies make it possible to directly assess the function of the cellular contractile apparatus in both healthy and diseased muscle, and are ideally suited to study muscle function at a cellular level. In order to elucidate both the advantages and the shortcomings of single fibre contractility studies, a brief stepwise explanation of the technique is warranted:

i. Bundles of muscle fibres are chemically permeabilised. This step is necessary to allow the different solutions to freely enter the fibre without restriction from the sarcolemma. For example, *in vivo*, intracellular Ca^{2+} is made available in response to an action potential that is sensed by the dyhidropyridine receptor (DHPR) on the T-tubule, leading to fast Ca^{2+} release from the sarcoplasmic reticulum via ryanodine receptor opening. As the latter process cannot be accomplished in resected muscle fibres, the membrane must be permeabilised to allow Ca^{2+} influx based purely on a concentration gradient.
ii. A single muscle fibre is dissected from the bundle and attached to aluminium clips at both ends (Figure 9).
iii. The permeabilised fibre is suspended between a lever arm connected to a motor on the one side and a force transducer on the other and bathed in a solution that allows the fibre to be stretched without opposing forces from the contractile proteins.

iv. The fibre is sequentially submerged in different solutions (e.g. activating solution) and the contractile apparatus exposed to the constituents of the solution, leading to either contraction or relaxation.
v. The contractile force generated by the fibre is recorded by the force transducer.

The technique has a number of advantages:

i. The factors that complicate the assessment of individual muscle function *in vivo* (e.g. muscle fibre orientation, the presence of endomysial connective tissue due to chronic disease, variation in motor unit recruitment, or muscle co-activation) are circumvented in single fibre studies.
ii. On a cellular level, the technique enables the functional assessment of the contractile apparatus in isolation. This is accomplished by directly providing the contractile apparatus with the constituents required for contraction (e.g. calcium, ATP), thereby circumventing processes such as neuromuscular transmission, ATP production and Ca^{2+} influx. When normalised to muscle fibre diameter, the contractile force produced by a fibre therefore provides a direct assessment of the function of the contractile apparatus.

These factors make single muscle fibre contractility studies a valuable tool to investigate the mechanisms of muscle dysfunction in muscle disorders. Together with motility assays, it is the only technique that can reliably assess qualitative aspects of muscle function.

3.2 Synopsis of current literature

Single fibre contractility studies have been utilised in many studies of both healthy individuals as well as people with different disorders that may affect muscle strength, including systemic disorders and disorders limited to the neuromuscular system. In healthy individuals, these include exploration of the effects of sex,[75] age,[75] exercise,[83-85] and even zero gravity environments during prolonged space flight.[86] Neurological disorders investigated by means of single fibre contractility studies include myopathic disorders,[18-20,87] neurogenic disorders,[88,89] and long-term paralysis due to chronic spinal cord injury.[90] In the case of IIMs, the possibility of impaired contractility has not been adequately explored.

3.2.1 *Assessment of single muscle fibre function in IIM*

To date, only a single study evaluating *in vitro* single muscle fibre function in untreated DM has been performed.[46] Five patients were included, and compared with healthy controls. No differences in SF of type I fibres were found between the two groups, although SF of type IIa fibres was greater in patients with DM than in controls. Furthermore, type I fibres of patients with DM had a higher unloaded shortening velocity ($V_{0)}$ than those of healthy volunteers. Given these findings, it was proposed that the force generating capacity and shortening velocity of the remaining (surviving) muscle fibres are preserved in DM.

However, this study had some shortcomings. Firstly, and most importantly, the biopsied muscles in patients with DM were either of normal strength or only mildly weak (median MRC grade 4+/5, range 4-5). Impaired contractility as a sole or contributing

cause of weakness cannot be reliably excluded in the absence of significant clinical weakness in the biopsied muscles, as the muscle tissue obtained from such a muscle may not be representative of the processes leading to weakness. Secondly, not all biopsies were taken from the same muscle: all control biopsies were from the vastus lateralis muscle, while DM biopsies were from the biceps or deltoid muscles. Although studies comparing contractile force between different muscles, have not been performed, it cannot be assumed to be similar. Ideally, therefore, muscle biopsies should come from the same muscle in both groups. Muscle fibre contractility in IIMs therefore remains largely unexplored. The findings in this study have not been duplicated, and, as explained in chapter one and in section 2.2 below, data from animal studies suggest that contractility is indeed impaired in response to the presence of inflammatory mediators.

3.2.2 *Mechanism of muscle fibre dysfunction in animal models of IIMs*

As referred to in chapter one, exogenous TNF-α has been shown to decrease contractile force in diaphragmatic and limb muscles in animal models, both *in vivo* and *in vitro*.[42,44,43] A single study evaluated contractility in chemically permeabilised murine diaphragmatic single muscle fibres after intraperitoneal injection of TNF-α.[91] TNF-α decreased the force generated by myofibrillar proteins, acting via the TNF type I receptor subtype. The underlying mechanism appears to be mediated by TNF-stimulated oxidants, either reactive oxygen species (ROS), nitric oxide (NO), or both. No comparable studies on human skeletal muscle have been performed.

3.3 Purpose of the study

3.3.1 *Aims and objectives*

The aim of the study was to determine whether the function of the contractile apparatus of muscle fibres from patients with IIMs is affected by the disease process. In order to investigate this question, *in vitro* single muscle fibre contractility studies were performed to assess the maximum contractile force of the fibres from patients with IIMs and compare the results to those from healthy controls. The specific objectives of the study were:

i. To measure and compare the contractile force of single muscle fibres from patients with treatment-naïve IIMs to that of fibres from healthy volunteers.
ii. To correlate the contractile properties from the single fibres with clinical features (weakness) in patients with IIMs.

3.3.2 *Hypothesis*

The hypothesis is that weakness in IIMs is related not only to a decrease in the number of muscle fibres (i.e. a structural abnormality due to inflammatory necrosis), but also to an impairment in the contractile function of individual muscle fibres (i.e. a functional abnormality). Therefore, it was hypothesised that single fibre contractility studies would reveal a decrease in both maximum force (P_0) and SF of muscle fibres from patients with IIMs. It was furthermore hypothesised that the reduced force production would correlate with weakness documented on clinical examination.

3.4 Methods

3.4.1 *Sources of muscle biopsy specimens*

Muscle biopsy specimens were obtained from cases and controls as described in chapter two.

3.4.2 *Specimen acquisition, storage and preparation*

The acquisition and storage of muscle samples were described in chapter two. Prior to analysis, each of the stored samples was thawed briefly in PBS at 37°C for 1 minute and divided into small bundles, each consisting of 20-40 muscle fibres. These bundles were then submerged into skinning solution containing 50 % glycerol (pH 7.00), and stored at 4°C for 24 hours. After 24 hours, the skinning solution was replaced with fresh skinning solution and subsequently stored at -20°C until analysis.

3.4.3 *Equipment*

Single fibre contractility assays were performed on an Aurora Scientific 1400A permeabilised fibre test system (Aurora Scientific, Ontario, Canada). The system essentially consists of a force transducer and a motor to which each end of the fibre was attached, and a bath plate containing baths for different solutions into which the fibre is submerged (Figure 8). Details about the different solutions utilised in the study is provided in APPENDIX A.

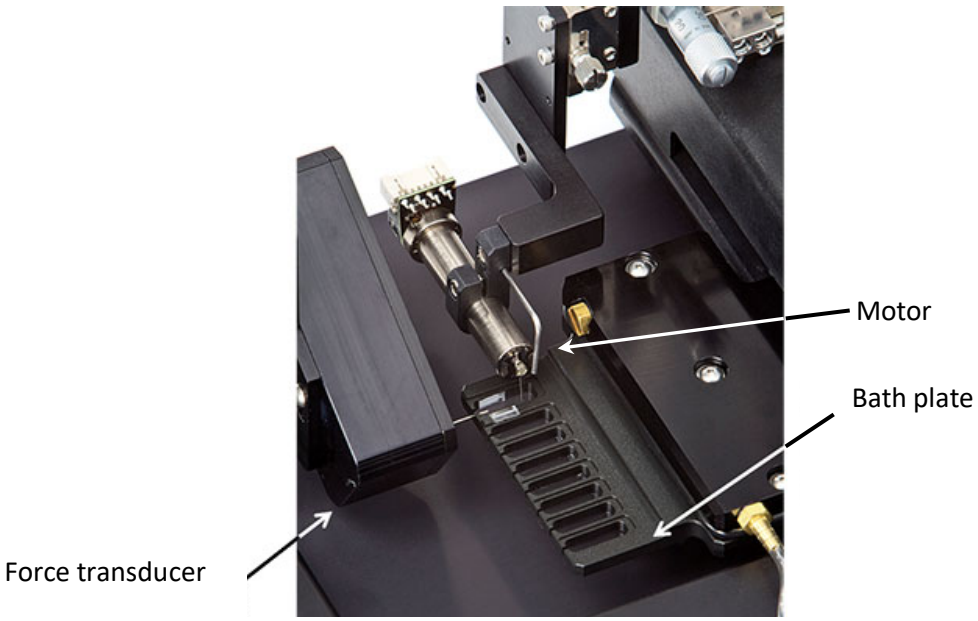

Figure 8. Components of the permeabilised fibre test system.

In order to attach each end of the fibre to the force transducer and motor, respectively, the fibre needs to be attached to two aluminium T-clips (Figure 9). Due to the exorbitant cost of commercially available T-clip templates, T-clips were made from aluminium kitchen foil based on previously described techniques, with some modifications.[92,93] For an illustrational video on the procedure, see: http://www.myolab.co.za/about-me/technology/technology-contraction/.

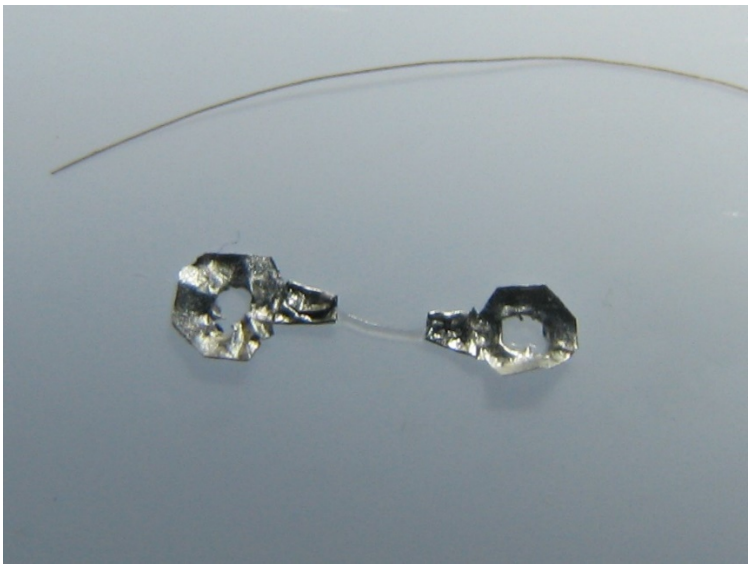

Figure 9. A muscle fibre attached to aluminium clips is shown next to a human hair for size perspective.

3.4.4 *Measurement of maximum force*

Contractile properties of skinned single fibres were analysed based on the methods described by Kohn et al.[94] All measurements and pH adjustments were performed at 12˚C. The volumes and concentrations of all solutions for the single fibre contractile properties were calculated using the computer program described by Dweck et al.,[95] based on that first published by Fabiato and Fabiato.[96]

On the day of the experiment, single fibres were dissected under a stereomicroscope and attached to aluminium T-clips (for an illustrational video on the dissection and attachment of muscle fibres, see: http://www.myolab.co.za/about-me/technology/technology-contraction/). The single fibre was then attached to the force transducer and motor and submerged in a relaxing solution containing 5 mM EDTA, 20 mM Imidazole pH 7.00, 5.08 mM ATP, with a total ionic strength of 180. The –log concentration of free ions amounted to: pMg 3.00, pCa 9.00 and pATP 2.40. After

mounting, the fibre was allowed to equilibrate in the relaxing solution for approximately 5 minutes. During this time, the fibre was stretched to obtain a sarcomere length of between 2.40 and 2.70 μm, the optimal sarcomere length to produce maximum contractile force. Digital images were recorded at 40x, 100x and 200x magnification for the determination of fibre length, diameter and sarcomere spacing, respectively, using a camera attached to the microscope (Figure 10). The fibre dimensions were determined using pre-calibrated software (AxioVision, Zeiss Inc., Germany). It was assumed that fibres have a circular shape, and CSA was determined from the diameter of the fibre using the equation $\pi [(0.8 \times \text{fibre diameter})/2]^2$, where 0.8 is to correct for an estimated 20% fibre swelling.[97] All subsequent operations were controlled by the software program of the permeabilised single fibre system.

Once equilibrated, the fibre was transferred to a bath containing a pre-activation solution (concentrations listed as above for relaxing solution, but with 0.5 mM EDTA, 14.5 mM creatine phosphate and 200 U/ml creatine kinase) for 30 seconds, where after it was transferred to an activating solution. The latter contained 5 mM EDTA, 20 mM imidazole pH 7.00, 5.08 mM ATP, 14.5 mM creatine phosphate and 200 U/ml creatine kinase. The –log concentration of free ions amounted to: pMg 3.00, pCa 4.50 and pATP 2.40. This concentration has been shown to elicit maximum contraction in human muscle fibres[85,73,98]. Progress of contraction was followed on the recording program, and once a steady state of maximum force was reached, the fibre was transferred back to the relaxing solution. Force was measured in millinewtons (mN). Next, the fibre was randomly assigned to either force clamp studies for determination of V_{max} (~70 % of fibres) or calcium sensitivity studies (refer to Chapters 4 and 5).

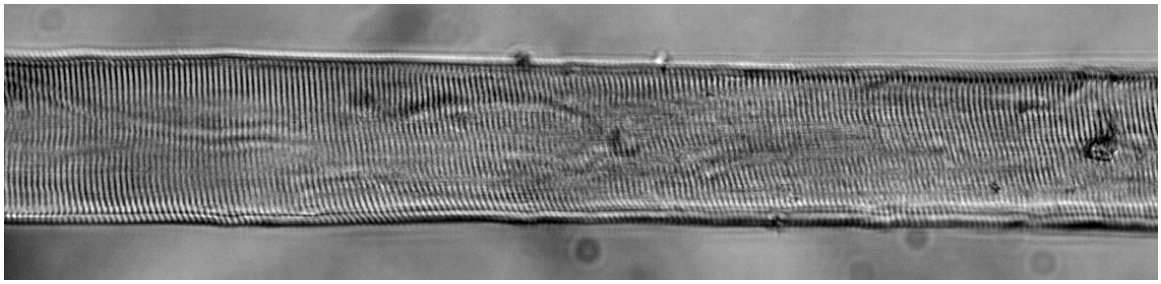

Figure 10. High-magnification photograph of a muscle fibre showing the striations, consisting of A-bands (dark) and I-bands (light).

3.4.5 *Determination of myosin heavy chain composition*

After force measurements were performed, each fibre was placed in a plastic microfuge tube containing 90 µl reducing sample buffer, and stored at -20˚C. The MyHC composition of each fibre was determined by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Kohn et al.[99] On the day of the experiment, 10 µl β-mercaptoethanol was added to each sample and the samples were heated at 95˚C for 5 minutes. The acrylamide concentrations were 4% (v/v) in the stacking gel and 7% in the separating gel. MyHC isoforms were identified using a homogenate that contained all three human MyHC isoforms (see Figure 11 for example). Approximately 25 µl of each single fibre preparation was loaded into each well, and 10 µl for the myosin homogenate.

3.4.6 *Statistical analysis*

SF was calculated as P_0 normalized to CSA and expressed as kN/m^2 (kilonewtons per square metre).

The D'Agostino & Pearson normality test was used to test data for normality of distribution. Because none of the data sets displayed a normal distribution, the non-parametric Mann-Whitney test was used to compare differences in means. Strength of association between variables was tested by means of the Spearman's Rank Correlation Coefficient. Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software, La Jolla, California). For all parameters, mean ± SD was calculated, unless stated otherwise. Statistical significance was set at $P<0.05$. Power calculations were performed using G*Power Version 3.1.9.2 (Erdfelder, Faul & Buchner, 1996).

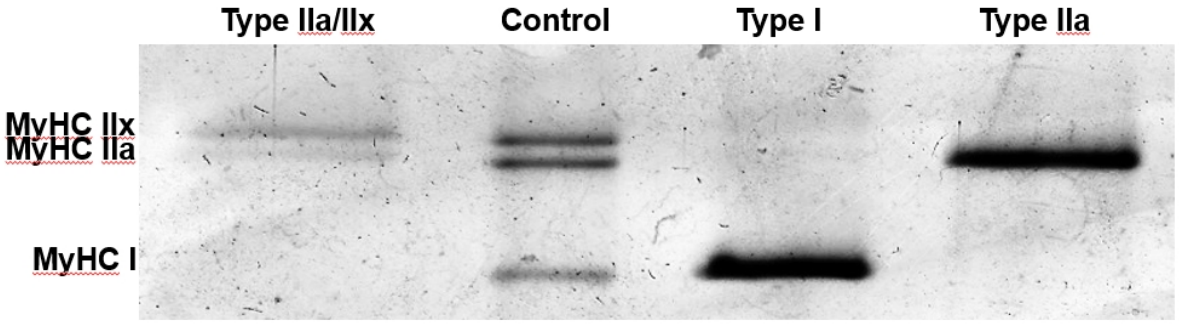

Figure 11. Electrophoretic separation of MyHC isoforms.

A muscle homogenate that contained all three MyHC isoforms (control) assisted in the identification of the single fibre isoforms.

MyHC: myosin heavy chain

3.5 Results

Participants included in this study consisted of six healthy controls (two male, four female) and five patients with IIMs (all female). The IIM group consisted of one patient with PM, two with DM, and two with NAM. The median MRC score for the IIM group was 4 (range 4- to 5). Overall, 178 single fibres were studied from patients with IIMs; this included 55 type I fibres, 98 type IIa fibres, six type IIx fibres, and 19 hybrid fibres (13

I/IIa, two I/IIx, four IIa/IIx). A total of 174 fibres, consisting of 96 type I fibres, 65 type IIa fibres, one type IIx fibre, and 12 hybrid fibres (10 I/IIa, one I/IIa/IIx, one IIa/IIx), were studied from the healthy control group.

3.5.1 *Analysis by group (IIM vs healthy controls)*

The contractile properties of IIM cases and controls were analysed for all fibre types combined (types I, IIa, IIx and hybrid), as well as for type I and IIa fibres separately. Type IIx and hybrid fibres were not analysed separately due to the small numbers of these fibres in the biopsies.

When all fibre types were combined, mean CSA of fibres from the IIM group was approximately 6% smaller than those from the control group (4 366 ± 2 337 μm^2 vs. 4 628 ± 1 749 μm^2; p=0.02). This difference was due to a smaller fibre size of type I fibres: when analysed separately, mean type I fibre CSA in IIM biopsies was 26% smaller than controls (3 342 ± 1 787 μm^2 vs 4 486 ± 1 703 μm^2; p<0.001), while type IIa fibre CSA was identical (4 796 ± 2 462 μm^2 vs 4 806 ± 1 815 μm^2; p=0.48), as illustrated in Figure 12. These findings are in contrast to those of comparative morphometric analysis described in chapter 2, which showed increased CSA for both fibre types when compared to published reference values and case series. The reason for this discrepancy in Type I fibre size between morphometric analysis and single fibre measurement is not clear, as selection of fibres for single fibre analysis is completely random.

Both maximum and SF measurements revealed statistically significant differences between cases and controls, not only for all fibres combined but also divided into fibre type. For all fibres combined, P_0 was 27 % lower in the IIM cases (0.33 ± 0.18 mN vs.

0.45 ± 0.17 mN; p<0.001, power = 0.99), while SF was 23 % lower (81 ± 38 kN/m^2 vs. 105 ± 45 kN/m^2; p<0.0001, power = 0.99). For type I fibres, P_0 was 48 % lower in the IIM cases (0.21 ± 0.09 mN vs. 0.40 ± 0.15 mN; p<0.0001, power = 1.00) and SF 24 % lower (73 ± 36 kN/m^2 vs. 96 ± 36 kN/m^2; p<0.0001, power = 0.96). For type IIa fibres, P_0 and SF were both 29% lower in IIM cases: P_0 was 0.37 ± 0.18 mN and 0.52 ± 0.20 mN in IIM and control fibres, respectively (p<0.0001, power = 0.99), while SF was 84 ± 36 kN/m^2 and 118 ± 48 kN/m^2 (p<0.0001, power = 0.99). These results are presented in Figure 13 and Figure 14.

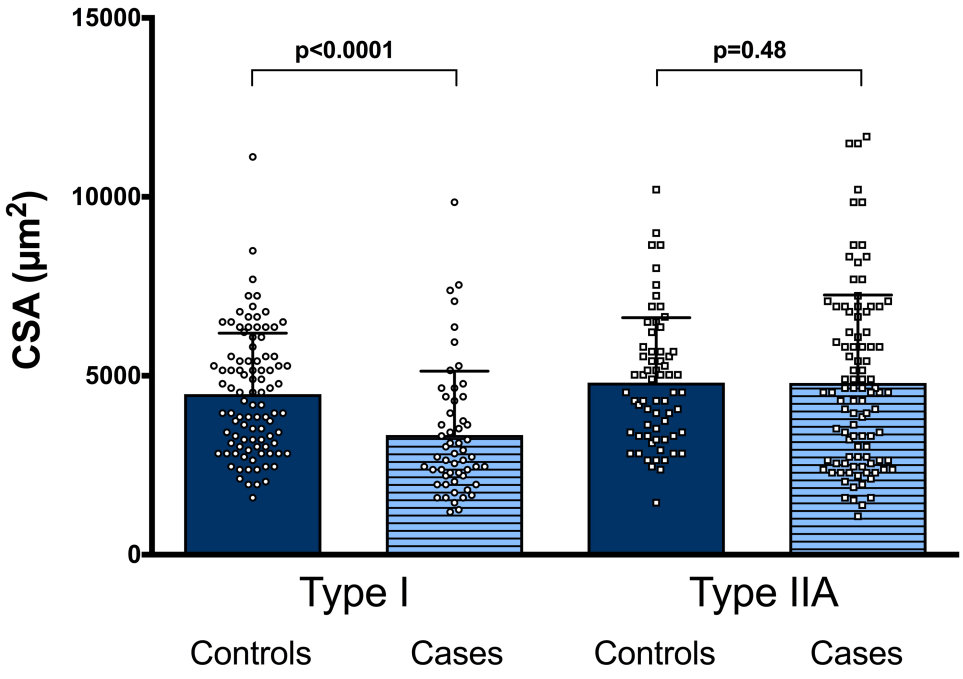

Figure 12. CSA of type I and IIa fibres (mean + SD).

CSA: Cross-sectional area.

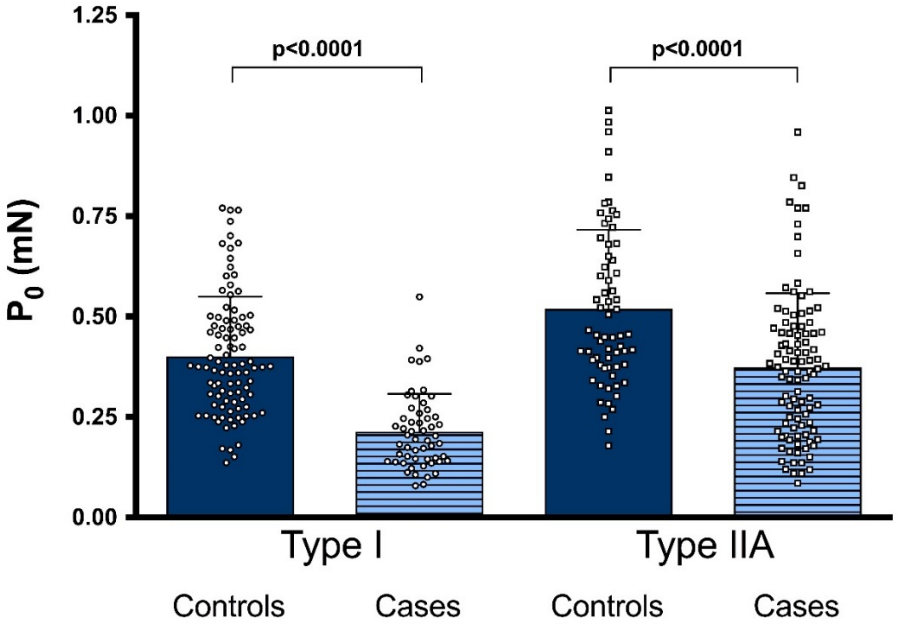

Figure 13. P_0 of type I and IIa fibres (mean + SD).

P_0: Maximum force.

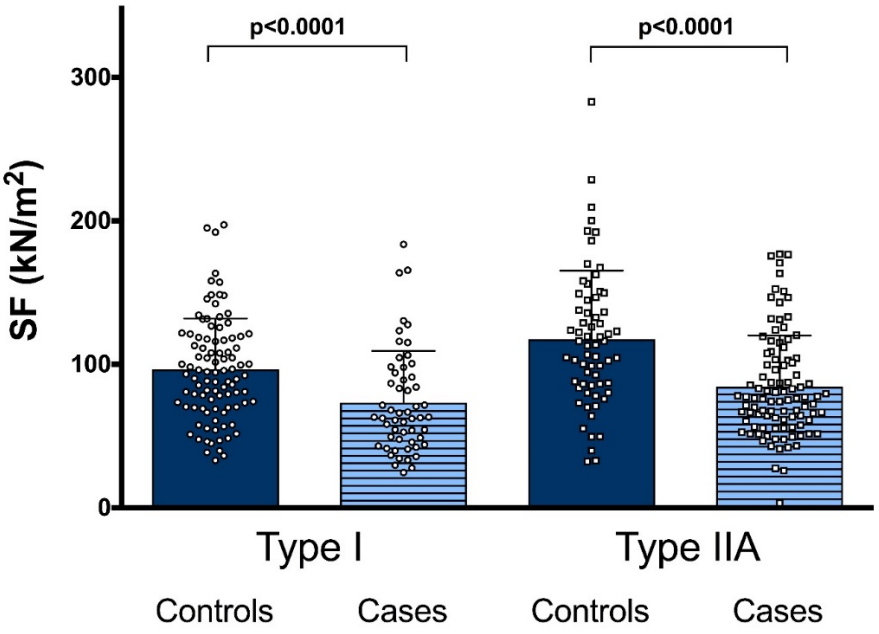

Figure 14. SF of type I and IIa fibres (mean + SD).

SF: Specific force.

3.5.2 *Analysis by diagnostic subgroup*

In order to investigate whether the findings seen in the IIM group as a whole were present in all subgroups (PM, DM and NAM) and not merely the result of a large effect size in one or two subgroups, fibres were also analysed in these different subgroups and compared to the healthy controls (whole group).

In the DM subgroup, type I, but not type IIa fibres, were significantly smaller in size. P_0 was significantly decreased for both fibre types, but SF was significantly decreased only for type IIa fibres. Interestingly, the type IIa fibres in the NAM subgroup were larger in size than in controls, while no significant difference was detected for type I fibres. P_0 was decreased for type I fibres, but identical for type IIa fibres (despite the increased CSA), while SF was significantly decreased for both fibre types. In the PM group, fibre size was significantly smaller for both fibre types, as was P_0. SF was significantly decreased for type I fibres, while there was a non-significant trend towards decreased SF for type IIa fibres. The results are shown in Table 8 and Table 8, while Table 10 provides a non-numerical summary of the findings.

	IIMs						CONTROLS
	DM	**p-value***	**NAM**	**p-value***	**PM**	**p-value***	
Number	14		15		26		96
CSA (µm²)	3 108 ± 1 702	<0.01	4 633 ± 2 325	0.88	2 724 ± 969	<0.0001	4 486 ± 1 703
***P₀* (mN)**	0.25 ± 0.13	<0.001	0.21 ± 0.08	<0.0001	0.19 ± 0.07	<0.0001	0.40 ± 0.15
SF (kN/m²)	88 ± 40	0.37	55 ± 30	<0.0001	76 ± 34	<0.01	96 ± 36

Table 8. Contractile properties of type I fibres analysed by diagnosis.

Values are mean ± SD. CSA: cross-sectional area; P_0: maximum force; SF: specific force; SD: standard deviation.

**p-values are calculated for each diagnostic subgroup compared to control group as a whole.*

	IIMs						CONTROLS
	DM	**p-value***	**NAM**	**p-value***	**PM**	**p-value***	
Number	59		21		18		65
CSA (μm²)	4 774 ± 2 410	0.63	6 407 ± 2 434	<0.01	2 990 ± 1 058	<0.001	4 806 ± 1 815
***P₀* (mN)**	0.35 ± 0.189	<0.001	0.52 ± 0.15	0.70	0.29 ± 0.09	<0.001	0.52 ± 0.20
SF (kN/m²)	77 ± 29	<0.001	90 ± 36	<0.01	100 ± 51	0.27	118 ± 48

Table 8. Contractile properties of type IIa fibres analysed by diagnosis.

Values are mean ± SD. CSA: cross-sectional area; P_0: maximum force; SF: specific force; SD: standard deviation.

**p-values are calculated for each diagnostic subgroup separately compared to control group as a whole.*

	DM		NAM		PM	
	Type I	**Type IIa**	**Type I**	**Type IIa**	**Type I**	**Type IIa**
CSA (μm²)	↓	↔	↔	↑	↓	↓
***P₀* (mN)**	↓	↓	↓	↔	↓	↓
SF (kN/m²)	↔	↓	↓	↓	↓	↔

Table 10. Summary of contractile properties of type I and IIa fibres analysed by diagnosis.

↓: Significantly lower compared to control group; ↑: Significantly higher compared to control group; ↔: No difference compared to control group.

3.5.3 *Contractile properties of individual IIM participants*

To investigate whether contractility data correlated with clinical weakness, mean P_0 and SF was calculated separately for each IIM participant (combined for all muscle fibre types), and plotted against MRC grading of muscle strength for knee extension, the action that *vastus lateralis* contributes to. Although strength was not formally tested in healthy controls, this was regarded as normal as, by definition, they had no complaints of weakness and no functional impairment. The Spearman's Rank Correlation Coefficient (r) was 0.674 (p=0.04), indicating moderately good correlation. The results are shown in Figure 15.

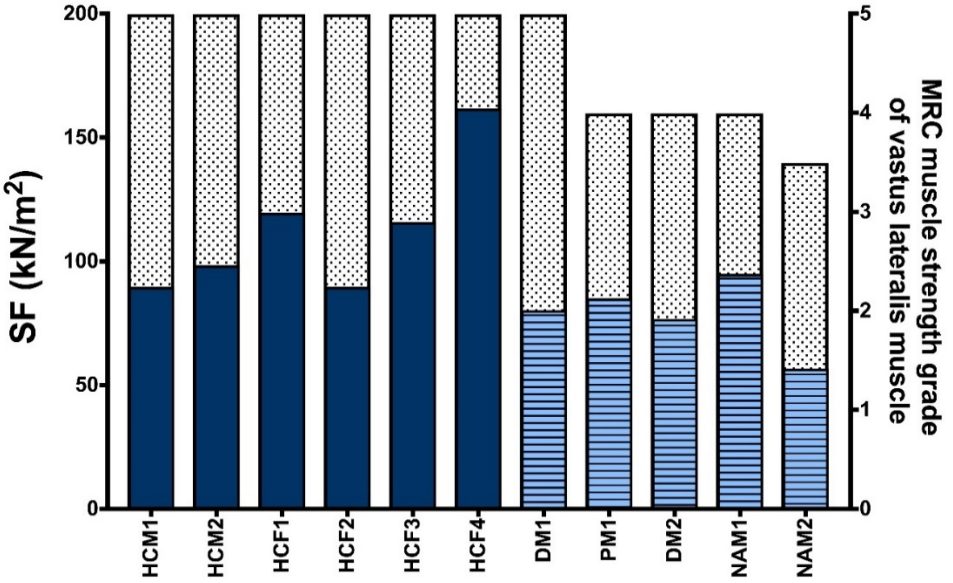

Figure 15. Mean specific force of all fibre types combined per participant (coloured columns, left Y-axis) and MRC strength grade of biopsied muscle (black-and-white dotted columns, right Y-axis).

HCM: Healthy control male; HCF: Healthy control female; DM: Dermatomyositis; NAM: Necrotising autoimmune myopathy; PM: Polymyositis.

3.6 Discussion

In order to investigate the mechanism behind weakness in IIMs, single fibre contractility studies were performed on muscle fibres from patients with IIMs, and compared to fibres from healthy controls. The findings of this study suggest that contractile force is impaired in IIMs. The impairment in contractile force can partially be explained by the fact that muscle fibre diameter is decreased in IIMs, leading to a decrease in maximum force production per fibre. However, there also appears to be an impairment in contractile force independent of a decrease in muscle fibre diameter, as evidenced by decreased contractile force after correcting for CSA (specific force, SF). The implication of this finding is that a functional impairment of muscle fibre contraction is present in IIMs, unrelated to a decrease in muscle fibre diameter.

The findings of this study provide a basis for further investigation into the possible mechanisms underlying weakness in IIMs. A number of different mechanisms have been proposed, but some of these cannot fully explain the findings. One such mechanism involves high mobility group box protein 1 (HMGB1), which may act via toll like receptor 4 (TLR4) to accelerate muscle fatigue in fibres by reducing the SR Ca^{2+} release.[100] This finding suggests that weakness and fatigue may result from impaired Ca^{2+} release from the SR. However, in the current study, the observed impaired contractility could not be explained by this phenomenon, as the Ca^{2+} required for contraction during single fibre studies is exogenously provided to the fibre through the activating solution and not released from the SR. Therefore, although impaired Ca^{2+} release leading to fatigue may partially explain fatigue in IIMs, it is unlikely to be an important mechanism in the development of impaired contractility. Similarly, these results do not support a prominent

role for abnormalities in energy metabolism, e.g. AMPD1 deficiency,[37] as contractility is impaired despite a sufficient supply of ATP by the activating solution. It also lends further support to the notion that the impaired enzyme activity described in Chapter 2 is a consequence of the disease process (e.g. related to inactivity) and is unlikely to contribute significantly to the development of muscle weakness. Therefore, although these mechanisms may contribute to weakness and fatigue, they do not explain the findings from this study of contractile dysfunction in muscle fibres from patients with IIMs.

In view of these findings, a more plausible explanation involves an interaction between one or more components of the inflammatory response and the contractile apparatus or its supporting structures. One such component is TNF-α, which has been shown to decrease contractile force of skeletal muscles in animal models.[42,44,43,91] In a study published in 1994, Wilcox and colleagues were able to show that infusion of TNF-α decreased diaphragm contractility in anaesthetised dogs by ~20 % within 3 hours and by ~50 % at 6 hours post-infusion.[44] Diaphragm contractility in this study was assessed by measuring force and shortening of the diaphragm *in vivo* during supramaximal phrenic nerve stimulation. It was therefore not possible to reliably localise the site of action of TNF-α, which could have been due to impaired transmission at the distal nerve, neuromuscular junction or along the sarcolemma, or due to impaired contractile function of the muscle fibres. In a subsequent study published in 1996, the same group performed *in vitro* assessment of excised hamster diaphragm contractility in response to incubation with high-dose TNF-α.[43] They were able to show an impairment in contractility in response to direct electrical muscle stimulation, thereby localising the site of action to muscle. In addition, they could illustrate partial blocking of this effect by indomethacin, an inhibitor of

cyclooxygenase, the rate-limiting enzyme in prostaglandin synthesis. This would suggest that the effect of TNF-α is mediated, at least in part, by cyclooxygenase products. In 2004, it was shown that TNF-α effects on muscle function were evident at the myofibrillar level in mice.[91] The euthanized animals received an intraperitoneal injection of TNF-α (or buffer for controls), and the diaphragms were excised 1, 2, 4, 24 or 48 hours after injection. Upon direct electrical muscle stimulation, maximal force was depressed by 25 % after 1 h, a response that persisted for at least 48 hours. Similarly, TNF-α decreased maximum SF of calcium-activated permeabilised diaphragm single muscle fibres, suggesting impaired contractility due to myofibrillar dysfunction. This effect appeared to have been mediated by oxidant activity, as intracellular oxidant activity was increased 1 h after TNF-α administration, and pre-treatment with Trolox (a hydrophilic antioxidant), diminished intracellular oxidant activity and abolished the decrement in SF of muscle fibres from TNF-α-treated animals. Li and colleagues (2000) came to a similar conclusion, showing decreased force production in mouse diaphragm fibre bundles in response to exogenous TNF-α.[41] The decreased force production was associated with greater intracellular oxidant levels and inhibited by incubation with the antioxidant *N*-acetylcysteine.

From a therapeutic perspective, if TNF-α is indeed an important role player in the development of contractile dysfunction, agents targeting this cytokine should theoretically result in clinical improvement. Monoclonal antibodies against TNF-α have recently become available, and some clinical data on one of these agents, infliximab, is available. This data is unfortunately limited to retrospective case series and a single, small randomized-controlled trial.[101-103] Although most cases showed a favourable response, some continued to worsen or discontinued treatment due to adverse events. However,

patients receiving infliximab in these series were all refractory to standard treatment, implying difficult to treat forms of the disease. More importantly, because disease duration in these patients was many years, it is highly likely that irreversible muscle damage had already occurred by the time infliximab was administered. Therefore, the apparent inefficacy of infliximab in some of the patients does not necessarily indicate an incorrect treatment target, but rather an irreversible stage of the disease. To reliably assess the efficacy of TNF-α inhibition, randomized controlled trials including treatment-naïve patients are required.

A number of different explanations for the impaired contractility should also be considered. Firstly, it is possible that the inflammatory process may have a deleterious effect on actin-myosin interaction, either by decreasing the amount of strongly bound interactions during maximal contraction, or a lower force generated by each individual interaction, or both. In a study evaluating the effect of age and immobilisation on single fibre contractility, D'Antona et al. (2003) were able to show a linear correlation between myosin concentration and loss of specific force.[74] This finding suggested that a lower number of interactions was responsible for the observed decrease in specific force of single fibres with aging and after immobilisation. In a different study of skinned fibres from elderly rats, Lowe et al. (2001), showed a reduced fraction of myosin heads in the strong-binding structural state with maximal activation.[104] Alterations in actin-myosin interactions and how they may be affected by the inflammatory response in IIMs have not been investigated, and warrant further consideration.

Another factor that may influence muscle fibre contractility is post-translational modification of myosin, of which the best studied example is phosphorylation. In

sarcomeric myosins, the Ca^{2+}/calmodulin-dependent skeletal muscle myosin light chain kinase (skMLCK) is responsible for phosphorylation, and phosphorylation sites are located on the regulatory myosin light chains (MyLC).[105] Phosphorylation of MyLC modulates Ca^{2+} / troponin-dependent force generation by promoting movement of the myosin heads out of the off-state.[106] However, impaired phosphorylation is unlikely to contribute to muscle fibre force generation for two reasons. Firstly, MyLC phosphorylation increases Ca^{2+} sensitivity and force at submaximal Ca^{2+} levels, and cannot explain the decreased force generation at maximal activation.[107,108] Secondly, and probably most importantly, although phosphorylation of MyLC has not been investigated in IIMs, other inflammatory disorders like inflammatory bowel disease (IBD) are associated with increased and not decreased MyLC phosphorylation.[109] Interestingly, this process appears to be mediated by inflammatory cytokines, in particular, TNF-α and IL-1β.[110]

In addition to the direct effects of the disease process on force production, the indirect effects, i.e. the role of muscle disuse/inactivity should also be considered. A number of studies investigated the effects of both long term and short term immobilization on contractile properties of skeletal muscle fibres, and a decrease in force was shown in some,[74,111,112] but not all of these studies.[90] However, it has to be noted that the extent of immobilisation was substantial in these studies (complete immobilisation, bed rest or chronic spinal cord injury), while all participants in the current study were still mobile, but had decreased activity due to weakness and fatigue.

Finally, the influence of the disease process on the amount of muscle fibre swelling should be considered. Although swelling was taken into account and corrected for, it is possible that the amount of swelling of IIM fibres exceeds that of control fibres.

Underestimation of the amount of swelling will lead to underestimation of the specific force. However, the CSA of IIM fibres in this study was decreased compared to controls. Although this does not necessarily exclude the presence of some intracellular oedema, it makes it highly unlikely. Furthermore, swelling of individual muscle fibres is not a reported feature on histological and ultrastructural assessment of inflammatory myopathy. In view of above, fibre swelling is unlikely to influence the results of this study.

During single fibre testing, muscle fibres from IIM cases were perceived to be substantially more fragile than fibres from controls, resulting in difficulty mounting the fibres in the aluminium clips without breaking them, and increased tearing of fibres during stretching to the desired sarcomere length. An attempt to quantify this was not made, as this was only observed during the course of the study. Although not likely, it is conceivable that the impaired force production may serve to protect the fragile fibre from irreversible damage. Assessment of the structural integrity of muscle fibres in IIMs should be considered in future research.

Study limitations

This study has a number of potential limitations. Firstly, it could be argued that the different disease entities included in the IIM group all have different pathological mechanisms. While this may be true, they also share a number of characteristics, some of which may be relevant to the context of the current study and provide sufficient justification to “lump” these entities together for the purpose of this investigation, as discussed in chapter one. Although the inclusion of sufficient numbers of patients with each of the separate disease entities would be ideal, such investigations are limited by

the rarity of the disorders and the time-consuming nature of the experiments. Furthermore, subgroup analysis of fibres from the different diagnostic subgroups, despite being limited in number, still revealed significantly impaired SF for one or both fibre types in each group (Table 8 and Table 8). A second limitation is incomplete matching for sex and age in the control group. Two of the six controls were male, while all five IIM cases were female, and the mean age in the IIM group was substantially higher. However, this is unlikely to have influenced the main results, as it has been shown that greater strength in men is primarily related to larger diameter of muscle fibres,[113] and both SF and normalised peak power are similar in men and women, as well as young and old.[75] One study did show decreased SF in elderly compared to young subjects (22% lower for type I and 16% lower for type IIa fibres), but the difference in median age between the elderly and young group was substantial (72.7 vs. 30.2 years).[114] In contrast, the median ages in the current study were 48 and 30 years for the IIM cases and controls, respectively, and the oldest participant in the IIM group was 60 years of age.

The reason for the finding of increased CSA of type IIa fibres from the NAM group is unclear. This finding has not been described before, and needs to be duplicated in a larger sample of patients with NAM. However, as discussed in chapter 2, there appears to be marked variation in CSA between individuals and between different studies, and caution is advised when interpreting fibre size data.

3.7 Conclusion

The results of this study suggest that maximal force generation of muscle fibres from patients with IIM are adversely affected by the disease process. The impaired

contractility correlates with weakness of the biopsied muscle, and could, at least partially, explain the presence of weakness in these disorders. Further studies are required to elucidate the pathological mechanism responsible for the development of the impaired contractility. In particular, the role of the pro-inflammatory cytokine TNF-α warrants further investigation in view of experimental animal data, as well as proteomics of individual fibres.

CHAPTER FOUR

4 FORCE-VELOCITY AND FORCE-POWER RELATIONSHIP OF SINGLE MUSCLE FIBRES FROM PATIENTS WITH IDIOPATHIC INFLAMMATORY MYOPATHIES

4.1 Introduction and background

In addition to measurement of force, contractile properties of muscle fibres can be further characterised by measurement of shortening velocity (*V*) at different loads/forces (including the unloaded state, i.e. *P* = 0 mN). When a muscle fibre contracts, the amount of force produced is dependent on the degree to which its ends are restrained. When an isometric contraction takes place (i.e. no shortening), force is maximal (P_0), while velocity will be zero, as the fibre length does not change, even though the myosin and actin still undergo vigorous cross-bridge cycling. If the ends are completely free, no force is produced and the fibre shortens at maximum velocity, which is termed unloaded shortening velocity (V_o). It is not possible to determine maximum shortening velocity if the ends of the fibre are attached, but the Hill equation is used to determine maximum velocity (V_{max}) when the fibres are attached. Between these two extremes, the relation between force and shortening velocity can be described as part of a rectangular hyperbola (Figure 16). This relation, first characterised by A.V. Hill in 1938, can be described by the equation $(P + a)(V + b) = (P_0 + a)b$, where *P* is the force during shortening at velocity *V*, P_0 is the

force during an isometric contraction, and *a* and *b* are constants, and is known as Hill's equation.[115] The curvature of the hyperbola is given by the value of a/P_0. Many investigators prefer determination of V_{max}, as it represents a closer simulation of physiological conditions, and enables the construction of velocity-force and power-force curves. It is important to note that, while the value of P_0 depends on the number of cross-bridges that are attached (and therefore the degree of filament overlap), the value of V_{max} reflects the rate of cross-bridge turnover and is independent of the number of cross-bridges in operation.[116-118,4]

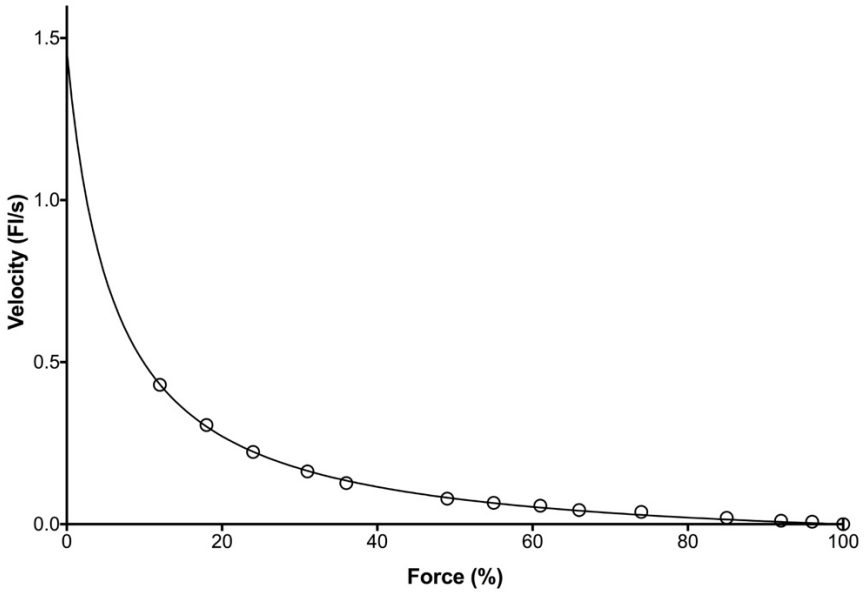

Figure 16. Force-velocity curve of a type I muscle fibre.

Force (x-axis) is expressed as percentage of P_0. The hyperbola meets the X-axis at P_0 and the Y-axis at V_{max}. The curvature of the hyperbola is given by the value of a/P_0. P_0: maximum force; V_{max}: maximum shortening velocity.

Measurement of V_{max} is more complicated than measurement of force. In order to determine V_{max}, conditions of zero load need to be recreated or simulated. Two main

techniques have been described, the slack test and the force clamp test. The slack test was first described by Edman (1979) and is performed by rapidly releasing the tension to zero during a contraction and measuring the time to the start of tension redevelopment.[118] This is repeated at different magnitudes of release, and the slope of the relation between size of the release and measured time represents the steady state shortening velocity at zero load (V_0), which is different from maximum shortening velocity at zero load (V_{max}). V_{max} is derived from the force-velocity curve by extrapolation of data obtained with force clamp testing. The difference between V_0 and V_{max} is not significant in slow twitch fibres, but V_0 is significantly greater than V_{max} in fast twitch fibres.[73] If both force and velocity is known, power output (W) can be calculated from the equation *power = force x velocity* ($W = P \times V$). From this equation, it follows that single fibre power can be improved by increasing force, velocity, or both. Therefore, when contractile force decreases, power output could theoretically be maintained by an increase in shortening velocity.

A single study compared maximal unloaded shortening velocity (V_0) of muscle fibres from patients with DM to fibres from healthy controls.[46] They found V_0 to be 33 % higher in type I fibres from patients with DM, while no difference was found in V_0 of type IIa fibres. They hypothesised that this may be a compensatory mechanism to maintain muscle power output in the face of the overall loss of force producing capacity which arose from muscle fibre atrophy. However, it should be noted that, in this study, single fibre maximal and specific force production in DM was not decreased compared to healthy controls, and therefore the increased V_0 should not necessarily be regarded as a compensatory mechanism.

4.2 Purpose of the study

4.2.1 *Aims and objectives*

The aim of the study was to determine whether the power output of muscle fibres from patients with IIMs is maintained despite the disease process. As contractile force is decreased in fibres from patients with IIMs (see Chapter 3), maintenance of power output would require an increase in shortening velocity. In order to investigate this, *in vitro* single fibre contractility studies were performed to assess the maximum shortening velocity (V_{max}) and calculate power output of single muscle fibres from patients with IIMs and healthy controls. The objectives of the study were:

iii. To compare the maximum shortening velocity of single muscle fibres from patients with treatment-naïve IIMs to that of fibres from healthy volunteers.

iv. To calculate and compare the power output of single muscle fibres from patients with treatment-naïve IIMs to that of fibres from healthy volunteers.

4.2.2 *Hypothesis*

It was hypothesised that, in an attempt to maintain power output in the presence of decreased force generating capacity (see Chapter 3), the maximum shortening velocity (V_{max}) of muscle fibres from patients with IIMs would be increased. However, since it can be anticipated that such an increase in shortening velocity will be limited by physical properties of the muscle fibres, it was furthermore hypothesized that this increase will only be able to partially compensate for the decreased force generating capacity, with a resultant decrease in power output, since $W = P \times V$.

4.3 Methods

The sources, acquisition and storage of specimens were described in Chapter 2, while the preparation of specimens and the solutions for single fibre experiments were described in Chapter 3. The procedures relating to the mounting, stretching, capturing of digital images, measurement of fibre dimensions (including fibre length, diameter and sarcomere spacing) and calculation of CSA and SF were described in Chapter 3. Force-clamp studies for determination of V_{max} were performed on the majority of fibres (~70 %), while the remaining fibres were used for calcium sensitivity studies (see Chapter 5).

4.3.1 *Determination of maximum velocity and power output*

Velocity measurement was performed on the Aurora Scientific 1400A permeabilised fibre test system (Aurora Scientific, Ontario, Canada). Experiments were performed based on previously reported protocols.[73,94,119] After measurement of P_0, as described in Chapter 3, the fibre was transferred back to the relaxing solution and allowed to relax and regain its initial length. Each fibre was then subjected to a series of isotonic force clamps to construct force-velocity curves. First, maximum activation in pCa 4.5 solution was again obtained, after which the fibre was subjected to a set of four successive force clamps, each clamp lasting 150 ms. For example, for the first force clamp set, the force was held constant at 80 % of P_0. After 150 ms, the fibre was allowed to shorten to the next pre-programmed isotonic load (55 % of P_0), and the process and data recording was repeated. After a further two force clamps (30 % and 10 % of P_0), while the fibre was still submerged in activating solution, it was subjected to a 2 ms shortening to 50 % of its original length, and then immediately re-stretched within 1 ms to its original length. This re-stretching procedure aided in preserving the fibre integrity when

subjected to the next force clamp series.[120,121] Following this, the fibre was transferred back to the relaxing solution and again allowed to relax and regain its initial length. The process was repeated 3 times at different isotonic loads, yielding a total of 16 force clamps in 4 sets (Table **9**). After measurements were performed, each fibre was placed in a plastic microfuge tube containing 90 µl sodium dodecyl sulphate (SDS) buffer, and stored at -20˚C. Determination of MHC composition was described in Chapter 3.

For each fibre, the shortening velocity was determined from the last 50 ms of each force clamp (length change), from which the slope was plotted against the force (% of maximum). A non-linear regression curve using the Hill-equation $(P+a)(V+b)=(P_0+a)b$ (where P is force, V is velocity, P_0 is maximum force and a and b are constants) was fitted to the data points. Maximum shortening velocity (V_{max}), expressed as FL/s (fibre length per second) was calculated by extrapolating the data back to 0 % force (see Figure 16). Power output (W) was calculated from the shortening velocity and specific force parameters using the formula power = force x velocity ($W = P \times V$) and expressed as kN/m^2.FL/s. These values were used to construct composite velocity-force and power-force curves, and ultimately determine maximum power output (W_{max}) for each fibre, as well as optimal force (force at which W_{max} is developed, P_{opt}), expressed as percentage of P_0 ($P_{opt} \times 100/P_0$). Force clamp measurements were excluded from analysis if the force at maximal activation during the initial phase of the clamp dropped below 90 % of P_0. In addition, measurements obtained at less than 10 % of P_0 were also excluded from the analysis. Because three of the force clamps were performed at forces less than 10 % of P_0 (see Table 9), this yielded a maximum of 13 velocity data points. Fibres that broke during any of the force clamp sets were excluded from analysis.

Force clamp set	Sequence of isotonic loads (expressed as percentage of P_0)
No 1	80 %, 55 %, 30 %, 10 %
No 2	60 %, 40 %, 15 %, 2 %
No 3	75 %, 50 %, 25 %, 6 %
No 4	70 %, 45 %, 20 %, 4 %

Table 9. Isotonic load sequences of each set of force clamp measurements.

Sets were performed in the same order (1 to 4) for each fibre. Each set consisted of 4 isotonic force clamps, performed in descending order. Isotonic loads are expressed as percentage of P_0 (maximum force).

4.3.2 *Determination of MyHC composition*

The protocol for MyHC determination has been described in Chapter 3.

4.3.3 *Statistical analysis*

The D'Agostino & Pearson normality test was used to test data for normality of distribution. Because none of the data sets displayed a normal distribution, the non-parametric Mann-Whitney test was used to compare differences in means. Categorical data were analysed by means of the two-tailed Fisher's exact test. Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software, La Jolla, California). For all parameters, mean ± SD was calculated, unless stated otherwise. Statistical significance was set at $P<0.05$.

4.4 Results

Participants included four patients with IIMs (all female), and four healthy controls (two male). Participant details are summarised in Table 10.

The IIM group consisted of one patient with PM, two with DM, and one with NAM. In the IIM group, 27 of 120 (23 %) fibres broke during force clamp testing (7 type I and 20 type IIa) and were excluded from the analysis, while another 2 fibres were excluded from analysis because of insufficient or unreliable data points. Overall, 91 fibres were studied from patients with IIMs; this included 25 type I fibres, 58 type IIa fibres, and 8 hybrid fibres (4 I/IIa, 2 I/IIx and 2 IIa/IIx). In the control group, 19 from 129 fibres (15 %) broke and were excluded (13 type I, 1 type I/IIa and 5 type IIa), while 9 fibres were excluded because of insufficient or unreliable data points. A total of 101 fibres, consisting of 66 type I fibres, 27 type IIa fibres, and 8 hybrid fibres (6 I/IIa, 1 I/IIa/IIx, and 1 IIa/IIx), were studied from the healthy control group. The difference between the two groups with respect to fibre breakage was not statistically significant.

	GROUP	
	IIMs	**CONTROLS**
Age	47 (26-60)	33 (22-44)
Gender	4F	2M, 2F
Diagnosis	1 PM, 2 DM, 1 NAM	Healthy, recreationally active
Fibre number	91 (33 type I)	101 (74 type I)

Table 10. Participant details.

Age is reported as mean (range) in years. DM: dermatomyositis, PM: polymyositis, NAM: necrotising autoimmune myopathy, F: female, M: male.

4.4.1 *Specific force*

When SF calculations were restricted to the subset of fibres that are included in the force clamp testing examined in this chapter, the results differed from those described in Chapter 3, where all fibres were analysed. For the present data set, while SF was 29% lower in type IIa fibres from IIM cases than controls (90 ± 38 kN/m^2 vs. 126 ± 40 kN/m^2; p=0.0001, power = 0.97), there was no difference between type I fibres from cases (91 ± 40 kN/m^2) and controls (92 ± 33 kN/m^2; p=0.69, power = 0.05). This difference appears to be due to the fact that the type I fibres from IIM cases with the lowest SF were included in the group of fibres that were selected for Ca^{2+} sensitivity analysis. As can be seen in Table 13 (Chapter 5), SF of type I fibres was substantially lower (51%) in IIM cases as compared to healthy controls, whereas the difference was only 27% for type IIa fibres. As fibres were randomly selected for either force clamp testing or Ca^{2+} sensitivity, this is likely to be a chance finding.

4.4.2 *Maximum shortening velocity and power output*

Type IIx fibres and hybrid fibres were not analysed separately due to the small numbers of these fibres in the biopsies. For type I fibres, V_{max} was 122 % higher in the IIM cases (1.40 ± 1.50 FL/s vs. 0.63 ± 1.10 FL/s; p<0.0001), while W_{max} was 105 % higher (4.10 ± 5.20 kN/m^2.FL/s vs. 2.00 ± 1.30 kN/m^2.FL/s; p<0.001) compared to controls. For type IIa fibres, V_{max} was 159 % higher in the IIM cases (2.00 ± 1.30 FL/s vs 0.77 ± 0.51 FL/s; p<0.0001) and W_{max} was 50 % higher (9.00 ± 4.90 kN/m^2.FL/s vs. 6.00 ± 3.50 kN/m^2.FL/s; p<0.01) than control values. Figure 17 and Figure 18 provide a visual representation of the V_{max} values, while Figure 19 and Figure 21 illustrates the velocity-force relationships for both fibre types.

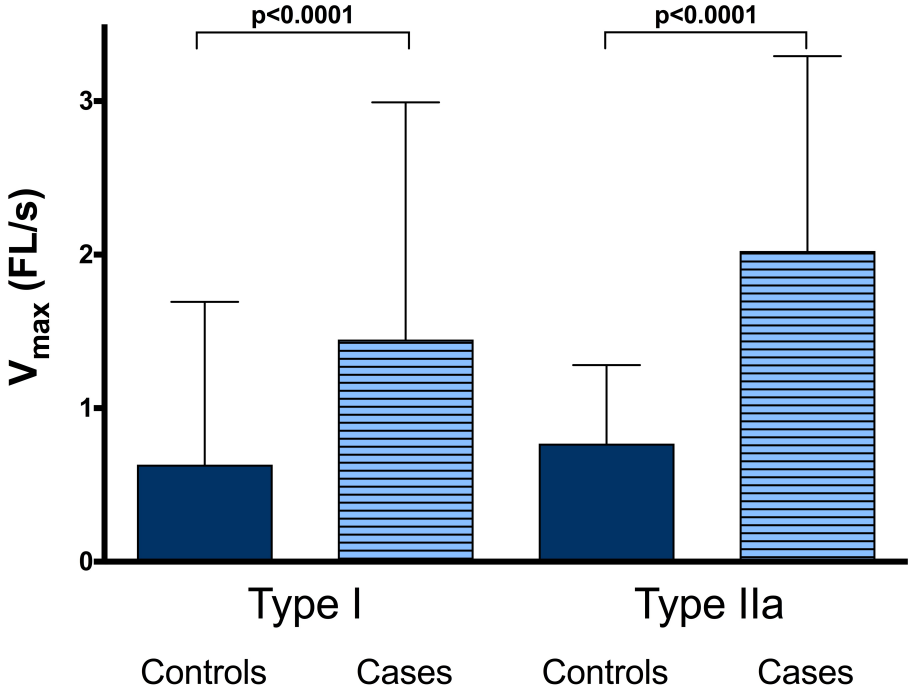

Figure 17. V_{max} of Type I and IIa fibres (means + SD).

V_{max}: maximum shortening velocity; FL/s: fibre length per second; SD: standard deviation.

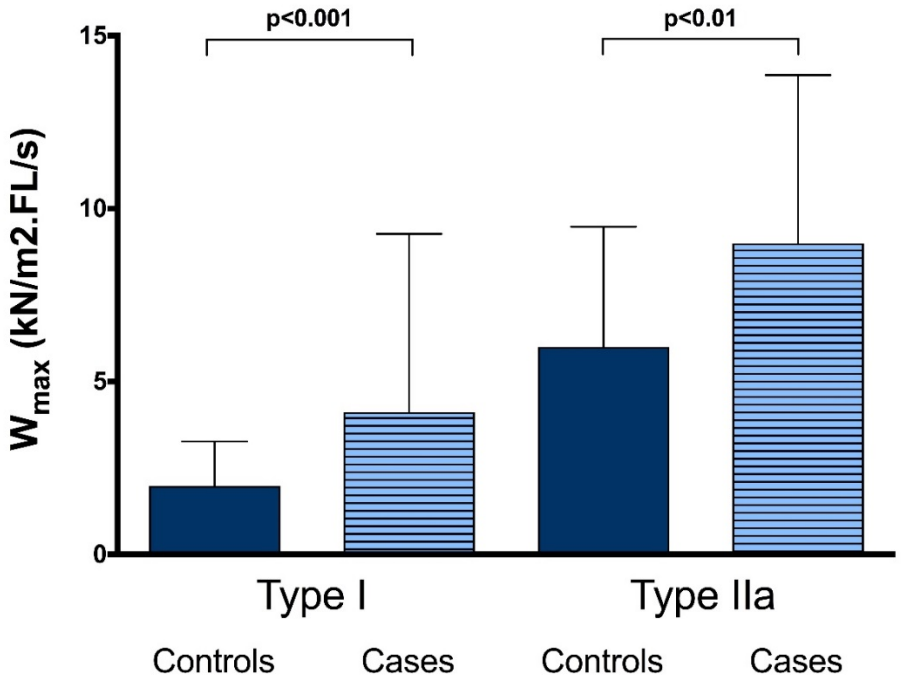

Figure 18. Power of Type I and IIa fibres (means + SD).

W_{max}: maximum power output, normalised to fibre CSA; SD: standard deviation.

4.4.3 *Optimal force*

W_{max} of type I fibres was obtained at 29 ± 9 % and 21 ± 7 % of P_0 for healthy controls and IIM cases, respectively. In the case of type IIa fibres, W_{max} was achieved at similar optimal forces as for type I fibres (27 ± 7 % and 22 ± 6 % for healthy controls and IIM cases, respectively). Fibres from IIM cases, both type I and IIa, were therefore able to develop maximum power output at lower relative force, with a resultant left shift in the power-force curve (Figure 20 and Figure 22). The difference was statistically significant for both type I ($p<0.0001$) and type IIa ($p<0.01$) fibres.

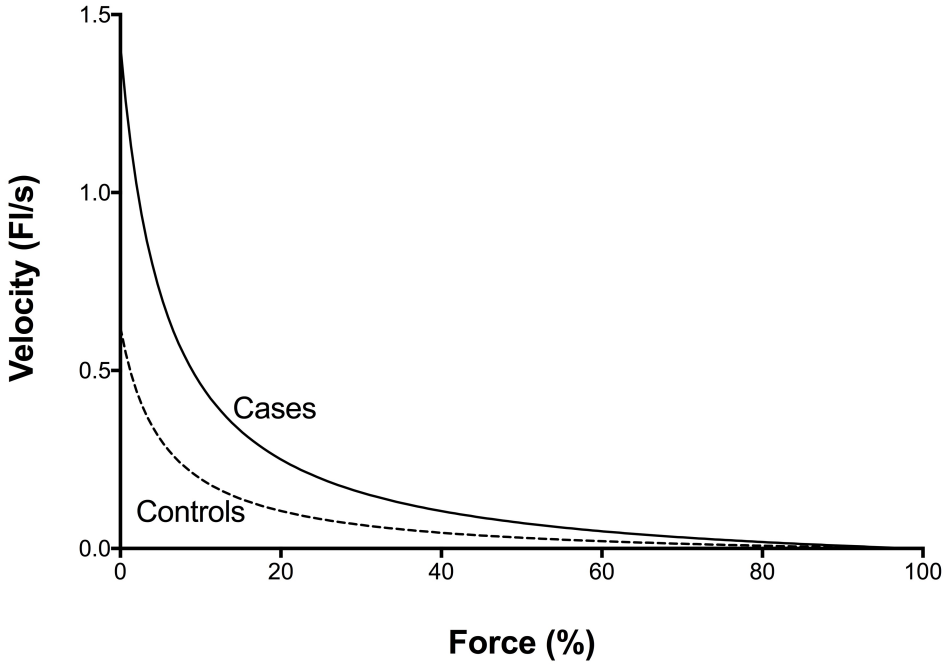

Figure 19. Velocity-force curves of Type I fibres of cases (solid lines) and controls (dotted lines).

Force is expressed as percentage of P_0 (maximum force).

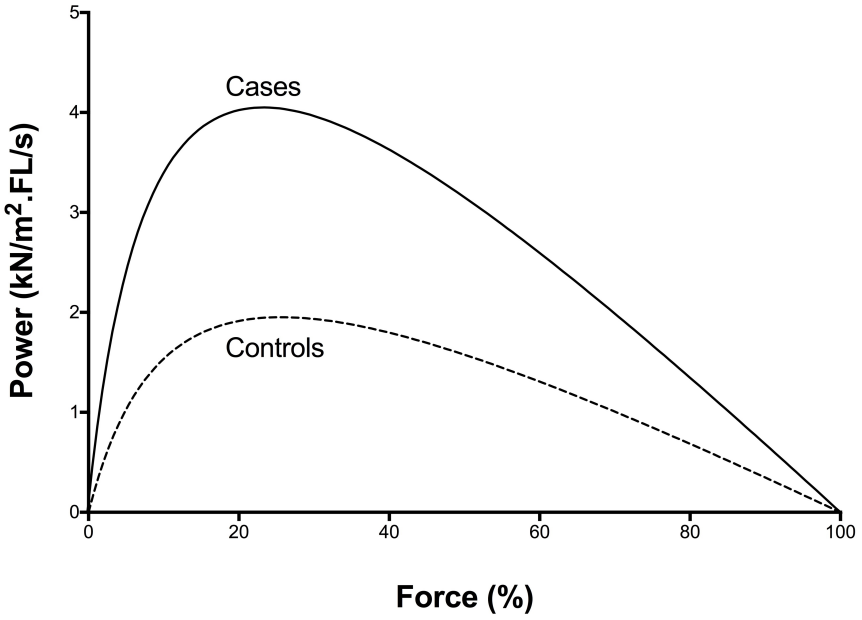

Figure 20. Power-force curves of Type I fibres of cases (solid lines) and controls (dotted lines).

Force is expressed as percentage of P_0 (maximum force).

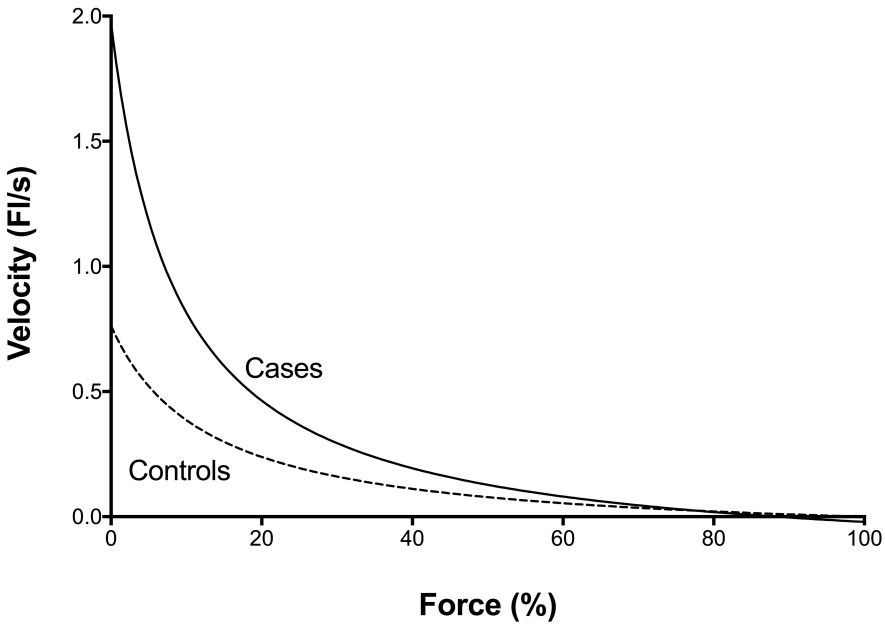

Figure 21. Velocity-force curves of Type IIa fibres of cases (solid lines) and controls (dotted lines).

Force is expressed as percentage of P_0 (maximum force).

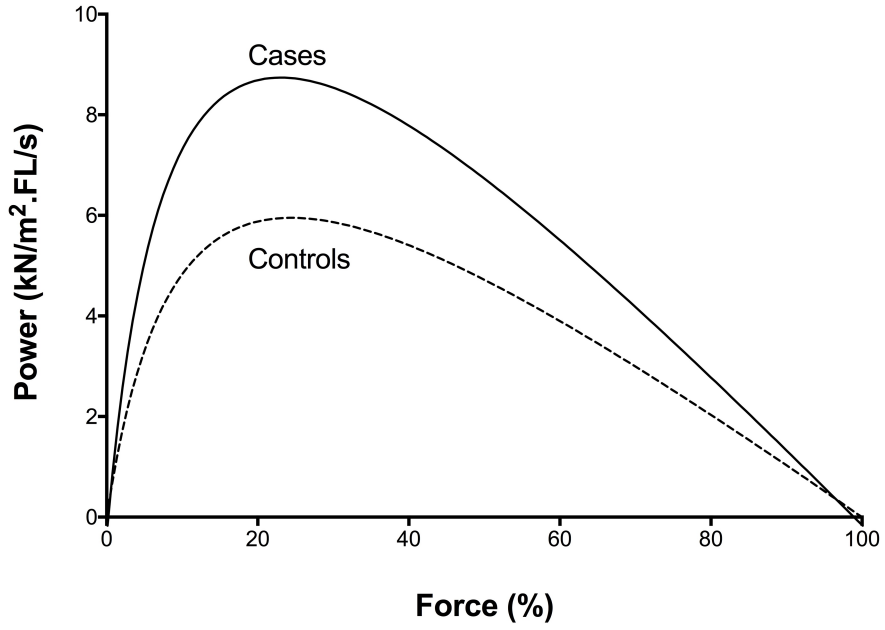

Figure 22. Power-force curves of Type IIa fibres of cases (solid lines) and controls (dotted lines).

Force is expressed as percentage of P_0 (maximum force).

4.5 Discussion

In order to test the hypothesis that shortening velocity of muscle fibres from patients with IIMs is maintained despite the disease process, *in vitro* single fibre studies were performed to assess force-velocity relationships and maximum shortening velocity of individual muscle fibres from patients with IIMs and healthy controls. The findings of the study suggest significantly higher maximum shortening velocity of fibres from patients with IIM. Furthermore, maximum power output was not only maintained, but was in fact higher compared to fibres from healthy controls. In addition, efficiency of muscle fibres from patients with IIM was increased, enabling them to develop peak power output at lower relative force.

If the hypothesis that the higher V_{max} is a mechanism to maintain power output is indeed correct, the mechanism behind this compensatory response is not clear. As mentioned in the introduction, V_{max} is dependent on the rate of cross-bridge turnover, which appears to be an intrinsic property of each individual actin-myosin interaction.[116-118,4]

One possible mechanism that may explain the observed findings relates to passive tension. Passive tension in muscle has traditionally been thought to arise from connective tissue between muscle fibres, as well as the non-contractile elements of the fibres (sarcolemma and sarcoplasm).[122] Collagen is a prominent component of fibrosis, which is often seen in IIMs.[123] Although it often becomes visible on routine histological examination only in more advanced disease, it is likely to be present from early in the disease process. Collagen appears to be one of the main contributors to passive tension

originating from connective tissue in cardiac muscle,[124] and is likely to have a similar effect in skeletal muscle. Although collagen is a component of the endomysium, skinned fibres contain small amounts of collagen. However, in a recent study of skinned fibres from *muscular dystrophy with myositis* (*mdm*) mice, mutant fibres contained 7.9 ± 3.5 % collagen vs. 0.3 ± 0.1 % in control fibres.[125] This finding suggests that collagen may contribute to mechanics at a fibre level, even though it is a component of the extracellular matrix. Recently, evidence has emerged that an important component of passive tension arises from the sarcomere, in particular the protein titin.[126] Whitehead et al. (2001) showed that passive tension in muscle rises after muscle injury due to repeated eccentric contractures. They suggested that this rise in passive tension was the result of injury-induced calcium-mediated contracture.[127] Furthermore, single muscle fibre studies in facioscapulohumeral dystrophy (FSHD) have shown that both passive force and calcium sensitivity were increased, while myofilament lattice spacing was decreased (suggesting titin stiffening).[20] The authors hypothesised that titin stiffening is a mechanism to compensate for muscle weakness in FSHD by increasing the calcium sensitivity of the sarcomeres. Although the pathogenesis of FSHD and IIMs is different, it is possible that similar compensatory mechanisms exist across the spectrum of muscle disorders, including the IIMs.

Interestingly, increased V_{max} has also been illustrated in nervous system conditions without direct muscle fibre damage, such as spinal cord injury and amyotrophic lateral sclerosis,[88,90] as well as chronic unloading of muscle, like prolonged bed rest[112,74] and space flight.[128] In the case of prolonged bed rest, the increased V_0 was associated with a decrease in maximum SF, whereas SF was not affected in spinal cord injury. Moreover,

in contrast to the findings in FSHD,[20] passive tension was not increased in muscle fibres from patients with spinal cord injury. Matters are further complicated by reports of increased V_0 in response to certain types of strengthening exercise, a contrasting physiological stimulus in terms of muscle activation.[119]

In addition to an increase in passive tension, there are other potential mechanisms to increase V_{max} which require consideration. Firstly, differences in MyLC isoform content and phosphorylation could theoretically affect V_0. In the rat, variability of V_{max} in fibres with similar MyHC content is related to MyLC isoform content, with higher velocity correlating with a higher alkali MLC3f content.[129,130] However, a similar effect of the MyLC isoform on shortening velocity in human fibres has not been demonstrated in a number of conditions including normal physiological conditions,[131] space flight,[128] endurance training[84] and aging.[74] Interestingly, in rats, the MyHC isoform that seems most sensitive to MyLC isoform variation is IIb. While this is the predominant isoform in rats, it is absent or only minimally expressed in human skeletal muscle,[8] which may partially explain the fact that maximum shortening velocity in humans appeared to be insensitive to MyLC variation. Post-translational modification of myosin, in particular phosphorylation of regulatory MyLC, also does not appear to influence maximum shortening velocity.[108,132]

Secondly, the influence of the mechanical performance of myosin molecules on shortening velocity should be considered. The sliding velocity of actin over myosin at zero load is dependent on the rate of cross-bridge turnover, and potentiation of this interaction could theoretically influence shortening velocity. The velocity at which myosin translocates actin filaments can be measured by means of the *in vitro* motility assay (IVMA). This method allows direct observation of the movement of a single fluorescently

labelled actin filament as it glides over a myosin-coated glass surface, and eliminates passive tension from the experimental set-up.[133] To date, no IVMA investigations of muscle fibres from patients with IIMs have been performed, but decreased sliding velocities have been demonstrated in diaphragmatic and cardiac muscle of dystrophic mice.[134,135] In addition, although immobilization was associated with increased maximum shortening velocity, it did not affect the actin sliding velocity as assessed by IVMA.[74] This assay would be invaluable to further investigate the mechanisms behind the increased maximum shortening velocities observed in the current study, as a normal motility assay will support the contribution of passive tension, and vice versa.

An interesting finding of this study is that power output of fibres from patients with IIM is not only maintained, but actually increased, suggesting that the increase in V_{max} is of a higher magnitude than the decrease in force generation. Despite this response, clinical weakness still develops, emphasising the relative importance of force production compared to shortening velocity in muscle function. It should also be noted that, despite the theoretical advantages of increased shortening velocity, it is entirely possible that the observed response is an unintended consequence, rather than a true compensatory mechanism. In order to clarify this, repeat single fibre force and velocity measurements, performed after resolution of weakness with treatment, will be informative. Decreased or normalisation of maximum shortening velocity, in parallel with increase or normalisation of specific force, will support the hypothesis of a compensatory mechanism.

Study limitations

This study has a number of potential limitations. Firstly, the same limitations discussed in the chapter on force measurement (Chapter 3) are applicable, namely the pooling of different disease entities in the IIM group and an incomplete matching for sex and age. However, as for force measurement, these limitations are unlikely to have significantly influenced the results, for reasons as discussed in Chapter 3. In addition, it should be noted that a few studies suggested a decrease and not an increase in maximum shortening velocity with age.[74,114] The higher median age in the IIM group in this study therefore makes the findings even more convincing.

4.6 Conclusion

The findings of this study suggest that compensatory responses to maintain power output occur in muscle of patients with IIMs. These responses include increased maximum shortening velocity as well as improved efficiency, and appear to be present in both type I and IIa fibres. The mechanism underlying this response is unclear, but may be related to increased passive tension, increased rate of cross-bridge turnover, or a different, as yet unidentified factor.

CHAPTER FIVE

5 THE RELATIONSHIP BETWEEN FORCE AND CALCIUM CONCENTRATION OF SINGLE MUSCLE FIBRES FROM PATIENTS WITH IDIOPATHIC INFLAMMATORY MYOPATHIES

5.1 Introduction and background

As discussed in chapter 1, contraction of muscle fibres is dependent on a sufficient supply of intracellular calcium (Ca^{2+}), which acts as a regulatory factor. *In vivo*, membrane depolarization leads to an influx of Ca^{2+} ions *via* voltage-gated Ca^{2+} channels, in turn leading to opening of Ca^{2+} channels (ryanodine receptors) in the SR (SR). This process ensures the large and rapid supply of Ca^{2+} required for contraction. Myosin-actin interaction, and hence the generation of force, is directly dependent on the Ca^{2+} concentration.[136-138] At very low Ca^{2+} concentrations, no force is generated, while steadily increasing Ca^{2+} concentrations lead to increased force development. However, when all Ca^{2+}-binding sites on troponin are occupied, this relationship exhibits a saturation effect, and increasing Ca^{2+} concentrations do not result in a further increase in force production.

Muscle fibre force production in response to different intracellular Ca^{2+} concentrations can be evaluated *in vitro* by means of single fibre Ca^{2+} sensitivity

measurements. These are accomplished by submerging single permeabilised muscle fibres into solutions containing different concentrations of free Ca^{2+} ions and measuring maximum force development at each concentration. Utilising this technique, the relationship between force production and Ca^{2+} concentration has been shown to follow a sigmoidal curve (Figure 23), with maximal force production at pCa 4.6.[139]

Ca^{2+} sensitivity of skeletal muscle is dependent on the complex formed by three troponin (Tn) subunits: inhibitory TnI, tropomyosin-binding TnT, and Ca^{2+}-binding TnC.[140] A study by Moss et al. (1986) suggested that TnC plays a central role in the force / pCa relationship of skeletal muscle fibres.[141] As opposed to the slow TnC isoform, which has one Ca^{2+} binding site, the fast isoform has two, which hypothetically confers the fibre a lower sensitivity to Ca^{2+}. It has also been shown that TnT plays a central role in Ca^{2+} sensitivity by regulating actomyosin ATPase activity.[142] Taken together, these findings provide a theoretical framework for the reported difference in Ca^{2+} sensitivity between type I and type II fibres.[143,84]

However, Ca^{2+} sensitivity of muscle fibres may also be influenced by a number of physiological factors. Higher temperatures decrease Ca^{2+} sensitivity (curve shifts to the right),[144,145] while plyometric exercise and osmotic compression lead to an increase in Ca^{2+} sensitivity (left shift of the curve).[143,146] The exact mechanism behind such changes in Ca^{2+} sensitivity is not clear. The increased Ca^{2+} sensitivity after plyometric exercise does not appear to be related to differential TnT isoform expression, but the role of other proteins of the contractile apparatus remains to be explored. A likely mechanism for the temperature dependence is an effect on the binding of Ca^{2+} ions to TnC,[144] while a

decrease in interfilament spacing may contribute to increased Ca^{2+} sensitivity in osmotic compression.[146]

In addition, pathological processes may potentially influence Ca^{2+} sensitivity in either direction. On one hand, it is possible that Ca^{2+} sensitivity may be improved as a compensatory mechanism in muscle disorders. For example, Lassche et al. (2013) studied permeabilised single muscle fibres from patients with FSHD, and found decreased maximal force generation and increased passive force and Ca^{2+} sensitivity in type II fibres.[20] They hypothesized that these changes represented compensatory mechanisms. As opposed to the viewpoint that Ca^{2+} sensitivity is compensatory, it is conceivable that certain disease processes might actually lead to a decrease in Ca^{2+} sensitivity by interfering with the response of the contractile apparatus to increasing intracellular Ca^{2+} concentrations. In IIMs, as shown in Chapter 3, sarcomeric force production appears to be decreased. Whether IIMs are also associated with changes in Ca^{2+} sensitivity has not been investigated. As discussed before, TNF-α appears to be a major role player in the development of weakness in these conditions and may exert this effect via a number of mechanisms, including reversible oxidative inhibition of myosin ATPase or TnC.[42] However, in animal studies, TNF-α decreased sarcomeric force production, but had no effect on Ca^{2+} sensitivity.[91] Thus, whether Ca^{2+} sensitivity of muscle fibres is altered in IIMs requires further exploration.

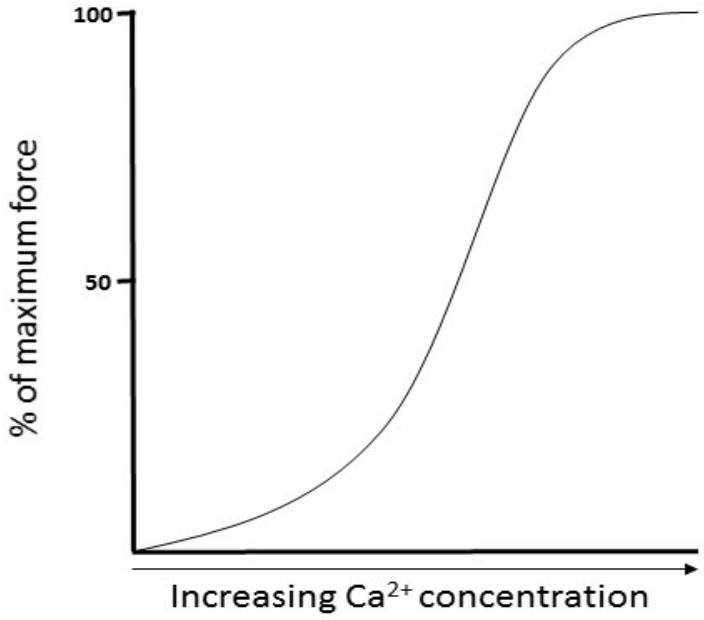

Figure 23. Force / Ca^{2+} relationship obtained by measuring maximum force production of permeabilised single muscle fibres at different Ca^{2+} concentrations.

5.2 Purpose of the study

5.2.1 *Aims and objectives*

The aim of the study was to determine whether the sensitivity to intracellular Ca^{2+} is altered in muscle fibres from patients with IIMs compared to healthy controls. In order to investigate this, *in vitro* single fibre contractility studies were performed to assess the force / Ca^{2+} relationship of single muscle fibres from patients with IIMs and healthy controls.

5.2.2 *Hypothesis*

As shown in Chapters 3 and 4, maximal force generation is decreased, while maximal shortening velocity and power output is increased in single muscle fibres from patients with IIMs. In view of similar findings in FSHD,[20] it was hypothesised that Ca^{2+} sensitivity will be increased in fibres from patients with IIMs.

5.3 Methods

The sources, acquisition and storage of specimens were described in Chapter 2, while the preparation of specimens was described in Chapter 3. The procedures relating to the mounting, stretching, capturing of digital images, measurement of fibre dimensions (including fibre length, diameter and sarcomere spacing) and calculation of CSA and SF were also described in Chapter 3.

5.3.1 *Solutions for force measurement*

The preparation of relaxing solution and maximally activating solution was described in Chapter 3. In summary, the relaxing solution contained 7 mM EGTA, 20 mM Imidazole, pH 7.00, 5.08 mM ATP, with a total ionic strength of 180 mM, and –log concentration of free ions amounting to pMg 3.00, pCa 9.00 and pATP 2.40. The maximally activating solution contained 7 mM EGTA, 20 mM imidazole, pH 7.00, 5.08 mM ATP, 14.5 mM creatine phosphate and 200 U/ml creatine kinase, with a total ionic strength of 180 mM. The –log concentration of free ions amounted to pMg 3.00, pCa 4.50 and pATP 2.40. In addition to these two solutions, six sub-maximally activating solutions, each identical to the maximally activating solution but with different Ca^{2+} concentrations ranging

from pCa 6.8 to pCa 5.1, were prepared. The different solutions were added to the wells of the permeabilised fibre test system in random order, as shown in Table 11.

Well no.	Solution	pCa
1	Relaxing solution	9.0
2	Submaximal activating solution	6.8
3	Submaximal activating solution	5.8
4	Maximum activating solution	4.5
5	Submaximal activating solution	5.1
6	Submaximal activating solution	6.2
7	Submaximal activating solution	5.4
8	Submaximal activating solution	6.0

Table 11. Distribution of the different solutions for force measurements at different Ca^{2+} concentrations in the wells of the permeabilised fibre test system.

The maximum and submaximal activating solutions differed from each other only with respect to the Ca^{2+} concentrations. Details about the different solutions are provided in APPENDIX A.

5.3.2 *Protocol for force measurements at different Ca^{2+} concentrations*

Calcium sensitivity measurement was performed on the same Aurora Scientific 1400A permeabilised fibre test system described in Chapters 3 and 4. Isometric force production at different Ca^{2+} concentrations was measured by transferring the muscle fibre segment rapidly between the different wells of the plate, each containing a different solution (Table 11). First, baseline force in the relaxing solution was recorded for at least 1 minute. The fibre was then rapidly transferred to well no 2, containing submaximal activating solution with a pCa of 6.8. The fibre was maintained in the activating solution

until a steady state of maximum force was reached, after which it was transferred back to the relaxing solution (well no 1), and allowed to relax and regain its initial length. The procedure was repeated for each of wells 3 through 8, and maximal developed force recorded at each pCa. The force developed at submaximal activating levels (relative force; P_r) was expressed relative to maximum force obtained at a pCa of 4.5 (P_0).

5.3.3 *Calculation of single fibre Ca^{2+} sensitivity*

For each fibre, the force – Ca^{2+} relationship was evaluated using an iterative nonlinear curve-fitting procedure based on the Marquardt–Levenberg algorithm. $pCa_{50\%}$, the Ca^{2+} concentration at which half-maximal activation occurs, was calculated based on the Hill equation: $P_r = pCa^n/(pCa_{50\%}^n + pCa^n)$, where n is the Hill coefficient, an indicator of the slope of the relationship.[147] All calculations were performed using GraphPad Prism version 7 (GraphPad Software, La Jolla, California). Occasionally, force values were obtained that were lower than the value at the preceding (lower) Ca^{2+} concentration. The reason for these outlying values was not clear, but no physiological explanation was evident, as force production increases with increasing Ca^{2+} concentration (up to pCa 4.5). These data points were therefore excluded from analysis because of the profound effect a single outlying value has on the shape of the curve. Fibres were excluded from analysis if less than 5 data points (excluding the zero value at pCa 9.0) could be used for the curve-fitting procedure.

5.3.4 *Determination of MyHC composition*

The protocol for MyHC determination has been described in detail in Chapter 3.

5.3.5 *Statistical analysis*

Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software, La Jolla, California). For all parameters, mean ± SD was calculated, unless stated otherwise. The D'Agostino & Pearson normality test was used to test data for normality of distribution. The unpaired t test with Welch's correction was used to compare means where data sets displayed a normal distribution, while the non-parametric Mann-Whitney test was used for data sets with non-normally distributed data. Statistical significance was set at $P<0.05$.

5.4 Results

Participants included consisted of five patients with IIMs (all female), and five healthy controls (one male). Participant details are summarised in Table 12.

	GROUP	
	IIMs	**CONTROLS**
Age	48 (26-60)	28 (22-44)
Gender	5F	1M, 4F
Diagnosis	1 PM, 2 DM, 2 NAM	Healthy, recreationally active
Fibre number	49 (17 type I)	41 (11 type I)

Table 12. Participant details. Age is reported as mean (range) in years.

DM: dermatomyositis, PM: polymyositis, NAM: necrotising autoimmune myopathy, F: female, M: male.

In the IIM group, 9 out of 58 (16 %) fibres (6 type I, 2 type IIa and 1 tipe I/IIa) were excluded from analysis because of insufficient data points, while 4 out of 45 (9 %) fibres (1 type I, 1 type IIa and 2 type I/IIa) were excluded in the control group. There was no difference in CSA between the two groups for any of the fibre types, but SF was significantly lower in type I and IIa fibres of IIM cases.

Ca^{2+} sensitivity analysis of type I fibres showed no difference in $pCa_{50\%}$ between the two groups (Table 13 and Figure 24) (power = 0.06). However, for type II fibres, as a whole, there was a small but statistically significant difference in mean $pCa_{50\%}$, which was 0.22 log units higher in IIM cases (power = 0.89), indicating an increase in Ca^{2+} sensitivity. Similarly, when type IIa fibres were analysed separately, $pCa_{50\%}$, was 0.24 log units higher in IIM cases (power = 0.75), with the difference remaining statistically significant (Table 13). The resultant left shift of the curve is illustrated in Figure 25. When comparing type I to type II fibres within each group, pCa was 0.12 log units higher in type II than type I fibres from IIM cases (p = ns), but 0.14 log units higher in type I than type II fibres from healthy controls (p = 0.03).

MyHC composition	IIM	Controls	p-value
Type I	(N=17)	(N=11)	
CSA (μm^2)	4150 ± 2109	3847 ± 1688	0.84
SF (kN/m^2)	58 ± 28	118 ± 37	<0.0001
$pCa_{50\%}$	5.53 ± 0.25	5.56 ± 0.23	0.73
Type II (all)	(N=24)	(N=29)	
CSA (μm^2)	4802 ± 2414	4656 ± 2171	0.81
SF (kN/m^2)	94 ± 45	108 ± 54	0.27
$pCa_{50\%}$	5.65 ± 0.31	5.43 ± 0.15	<0.01
Type IIa	(N=16)	(N=28)	
CSA (μm^2)	5113 ± 2572	4574 ± 2165	0.48
SF (kN/m^2)	80 ± 26	110 ± 54	0.04
$pCa_{50\%}$	5.66 ± 0.37	5.42 ± 0.15	0.02

Table 13. Force - pCa relationships of different fibre types of IIM cases and healthy controls.

Values represent means ± SD. MyHC: myosin heavy chain; CSA: cross-sectional area; SF: specific force; $pCa_{50\%}$: concentration at which half-maximal activation occurs. Because of a lack of data, no statistical analysis was performed to evaluate differences for type IIx and hybrid fibres.

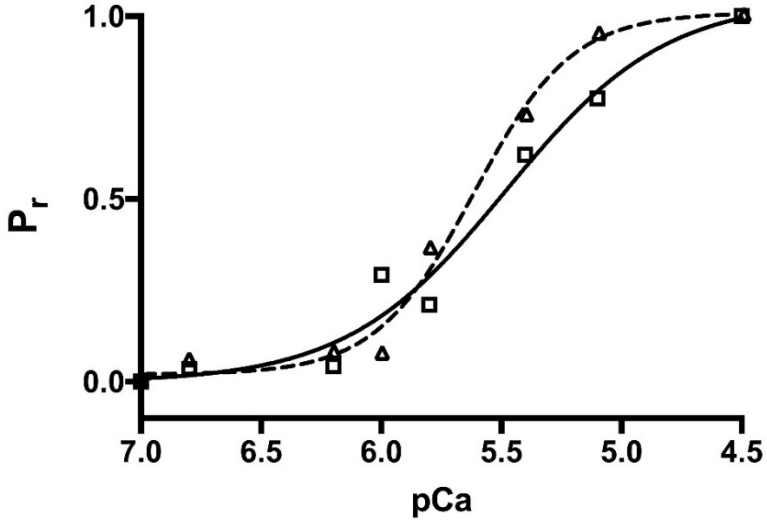

Figure 24. Force - pCa relationship of type I fibres of IIM cases (solid line) and healthy controls (dashed line).

The curves were constructed by calculating mean Pr (force developed at submaximal activating levels relative to maximum force obtained at a pCa of 4.5) at each pCa for each group, and performing an iterative nonlinear curve-fitting procedure.

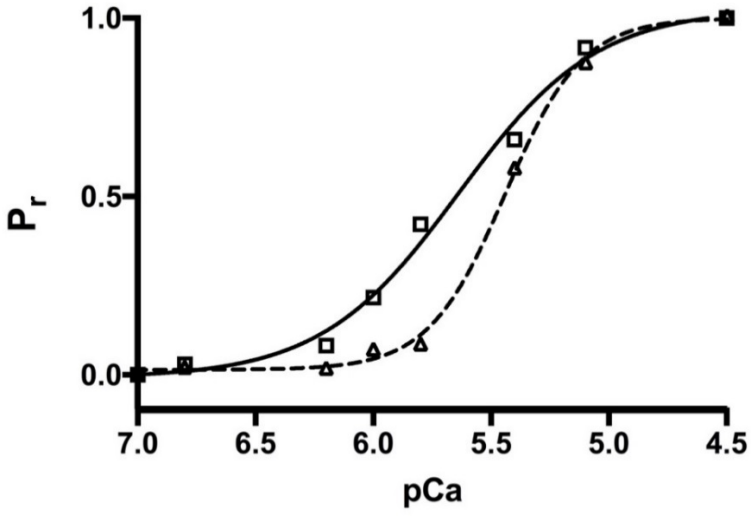

Figure 25. Force - pCa relationship of type IIa fibres of IIM cases (solid line) and healthy controls (dashed line).

The curves were constructed by calculating mean P_r (force developed at submaximal activating levels relative to maximum force obtained at a pCa of 4.5) at each pCa for each group, and performing an iterative nonlinear curve-fitting procedure.

5.5 Discussion

The relationship between intracellular Ca^{2+} concentration and force production during contraction has been studied in animal and human subjects under different physiologic conditions.[139,144,143,146] However, data on Ca^{2+} sensitivity in muscle disorders are limited to a single study of FSHD.[20] The present study investigated the force – pCa relationship in permeabilised muscle fibres from patients with IIMs compared to healthy controls. The findings of the study suggest a statistically significant increase in Ca^{2+} sensitivity of type IIa fibres of IIM cases, with a 0.24 log unit increase in $pCa_{50\%}$. Although this difference appears small, calculation of effect size reveals a Cohen's *d* of 0.85, which is regarded as a large effect size.[148] However, compared to the differences in shortening velocity and power output between IIM cases and controls described in Chapters 3 and 4, an increase of this magnitude is relatively minor, and unlikely to contribute significantly to the changes in velocity and power. Interestingly, in IIM cases, the Ca^{2+} sensitivity of type II fibres was higher than type I fibres (although the p-value was non-significant). This is in contrast to findings in healthy controls, both in the present study and previous reports, in whom Ca^{2+} sensitivity is higher in type I fibres.[143,84] This finding lends more support to the notion that only the Ca^{2+} sensitivity of type II fibres, in particular type IIa fibres, is altered in IIMs.

If the findings of the present study can be confirmed in a larger cohort, the mechanisms underlying such an alteration in Ca^{2+} sensitivity remains to be explored. In contrast to its possible effect on force production, TNF-α is unlikely to play a direct role in the modification of force – pCa relationships. Although chemically permeabilised muscle fibres from TNF-α treated animals had lower maximal Ca^{2+}-activated force, no change in

Ca^{2+} sensitivity was observed.[91] However, contractility studies were only performed on muscle up to 48 hours after TNF-α injection, and therefore a delayed effect of TNF-α on Ca^{2+} sensitivity could not be excluded. Another possible mechanism involves a decrease in myofilament lattice spacing, which was demonstrated in muscle fibres from patients with FSHD, together with an increase in Ca^{2+} sensitivity.[20] The authors ascribed both these findings to titin stiffening, based on previous reports.[149] Interestingly, the increased Ca^{2+} sensitivity seen with osmotic compression is also associated with a decrease in myofilament lattice spacing.[146,150] Based on the above, it is conceivable that decreased myofilament lattice spacing, regardless of the mechanism by which this is accomplished, may contribute to increased Ca^{2+} sensitivity. Whether myofilament lattice spacing is altered in IIMs has not been investigated and warrants consideration.

Any hypothesis regarding the potential mechanisms should take into account the fact that the increase in Ca^{2+} sensitivity was only evident in type II, and not type I fibres. A potential explanation for this involves the troponin complex (see *Introduction*). If inflammation can, directly or indirectly, lead to differential TnT or TnC isoform expression (i.e. slow TnT or TnC isoform expression in type II fibres instead of the fast isoform), it could lead to an increase in Ca^{2+} sensitivity. Such a mechanism would also account for the absence of an alteration in type I fibre Ca^{2+} sensitivity, as the slow isoforms are already present in this fibre type and Ca^{2+} sensitivity has therefore reached a ceiling.

Consideration should also be given to the possibility that the increased Ca^{2+} sensitivity is a consequence of immobilisation, and not directly related to the disease process. However, in a study investigating the effect of short-term (2 weeks) immobilisation on contractile properties of young and old individuals, Hvid et al. (2011)

found a decrease in Ca^{2+} sensitivity.[151] Similarly, Yamashita-Goto et al. (2001) showed decreased Ca^{2+} sensitivity after 2 and 4 months of bed rest (type I and II fibres were not analysed separately),[112] while Widrick et al. (1998) found decreased Ca^{2+} sensitivity in type I soleus muscle fibres after 17 days of bed rest (no type II fibres were analysed).[111] In contrast, Malisoux et al. (2007) found no alteration in Ca^{2+} sensitivity of any fibre type from long-term paralysed (spinal cord injury) patients.[90] The effect of immobilisation on Ca^{2+} sensitivity, if any, therefore appears to be the opposite of what the present study found in IIMs, excluding immobilisation as a potential mechanism.

Study limitations

This study has a number of potential limitations. Firstly, because Ca^{2+} sensitivity was only performed on a portion of the muscle fibres subjected to force measurement, the study lacks sufficient numbers and statistical power. However, despite this, the difference between the two groups was still statistically significant for type IIa fibres, with a relatively large effect size. A second limitation is incomplete matching for sex and especially age in the control group, with the mean age in the IIM group being substantially higher. Age difference between the two groups is, however, unlikely to have influenced the results. In a study by Lamboley et al. (2015), the difference in $pCa_{50\%}$ was ~ 0.05 log units lower in type IIa fibres from old (70 ± 4 years) than young (22 ± 3 years) participants.[152] In another study, Hvid et al. (2011) found no difference in Ca^{2+} sensitivity of type I or IIa fibres between young (24 ± 1 years) and old (67 ± 2 years) participants.[151] The effect of age on Ca^{2+} sensitivity therefore appears to be rather small or non-existent. If indeed present, Ca^{2+} sensitivity appears to be slightly higher in young individuals,

making the findings of the present study more robust, in view of the higher mean age of the IIM group.

5.6 Conclusion

Measurement of force – pCa relationship provides information on the sensitivity of the contractile apparatus to submaximal activating Ca^{2+} concentrations. The findings of this study suggest that the Ca^{2+} sensitivity of type IIa fibres from patients with IIMs is increased, while type I Ca^{2+} sensitivity is not altered. It is conceivable that the increased sensitivity of type IIa fibres serves as a mechanism to compensate for weakness and fatigue, but this hypothesis requires confirmation.

CHAPTER 6

6 INTERPRETATION OF FINDINGS AND FUTURE DIRECTIONS

6.1 Interpretation of findings

Although extensive research into the immunological and qualitative morphological characteristics of idiopathic IIMs has been performed, little is known about the mechanism(s) by which these result in the development of weakness. In contrast to muscular dystrophies, where inherited abnormalities of structural proteins are known to lead to dysfunction of the contractile apparatus or its supportive structures, the explanation for weakness in IIMs is relatively obscure. The aim of this project was to investigate the functional alterations and quantitative morphological changes in muscle from patients with IIMs, in order to gain a better understanding of the potential mechanisms underlying the development of muscle weakness and fatigue in this group of disorders.

As discussed in Chapter 1 (section 1.1.2), muscle weakness can theoretically develop either due to a decreased number of fibres contributing to contraction, or impaired contractile function of individual fibres. The different components of these two main mechanisms, and how the findings of this research project addressed each of them, is summarised below.

6.1.1 *Decreased number of fibres contributing to contraction*

The number of fibres contributing to contraction can be decreased either due to a loss of fibres as a result of necrosis, failure of conduction at the neuromuscular junction (NMJ), or loss of sarcolemmal excitability with resultant non-contraction due to the inability to generate an action potential. With regards to myofibre necrosis, the findings from this study (Chapter 2) and others suggest that the number of necrotic fibres in IIM biopsies (~ 1%) is insufficient to explain the degree of weakness.[39] Contrary to muscular dystrophies, there is also very little, if any, fibrosis visible on routine histological examination of IIMs, making extensive necrosis highly unlikely. Failure of conduction at the NMJ or the sarcolemma was not directly assessed in the present study, but the decreased force production illustrated by *in vitro* single muscle fibre contractility studies (Chapter 3) cannot be explained by conduction failure. One of the strengths of *in vitro* contractility studies is that it eliminates the role of the NMJ and sarcolemma by the direct intracellular provision of Ca^{2+} and ATP required for contraction, instead of relying on sarcolemmal depolarisation for Ca^{2+} release from the SR. Furthermore, motor nerve conduction studies, which assess the functional electrical integrity of the motor nerve, the NMJ, and muscle membrane, are typically normal in IIMs.[153] Based on these observations, a decrease in the number of contracting muscle fibres does not appear to be the major mechanism underlying weakness in IIMs, although a minor contribution cannot be wholly excluded.

6.1.2 *Impaired contractile function of individual fibres*

As discussed in Chapter 1, factors that hypothetically may lead to impaired contractile function of individual fibres include structural abnormalities of proteins involved in contraction, functional impairment of contraction (i.e. myofibrillary dysfunction), and deficient cellular energy production or calcium (Ca^{2+}) supply. Based on the findings of the present study, it is highly unlikely that cellular energy production or Ca^{2+} availability play a significant role in the impairment of contractile function. ATP, the main source of energy required for contraction, together with Ca^{2+}, are supplied in sufficient quantities in the activating solution. Because the sarcolemma is permeabilised, these chemicals are freely available to the muscle fibre during submersion into the activating solution. Therefore, if force production is shown to be impaired when performing contractility studies (Chapter 3), the impairment cannot be ascribed to availability of any of these chemicals. Although the activities of enzymes involved in cellular energy production were shown to be decreased (Chapter 2), this is more likely to be a consequence of the disease process (e.g. related to physical inactivity of the patients), rather than a contributing factor to weakness and fatigue. The activities of the four enzymes measured were similar to sedentary values, and the controls were in range with recreational and trained athletes.[68,154]

The impaired maximum force production illustrated in Chapter 3 can therefore best be explained by one or more abnormalities of the contractile apparatus. Whether these abnormalities are structural or functional is not clear, and hypotheses for both mechanisms could be generated. Currently, data from a number of animal studies favour a functional impairment mediated by TNF-α, probably via increased oxidative stress, as

discussed in Chapter 3. These data include impaired contractility within hours of TNF-α infusion or incubation,[42,44,43,91,41] partial blocking of this effect on contractility by cyclooxygenase inhibition,[43] increased intracellular oxidant activity in response to TNF-α administration,[91,41] and inhibition of both the oxidant activity and the effect on contractility by incubation with an antioxidant.[91,41] Although other mechanisms for impairment of contractile function, such as myosin ATPase inhibition and alteration of actin-myosin interactions are theoretically possible, there is currently no data to support such mechanisms.

In addition to maximum force production, the present study also investigated maximum shortening velocity, power output, and Ca^{2+} sensitivity of muscle fibres from individuals with IIMs. Both maximum shortening velocity and power output were significantly higher not only in type I but also in IIa fibres from IIM cases compared to controls, while Ca^{2+} sensitivity was increased only in type IIa fibres. While the significance of altered calcium sensitivity is less clear, the increase in maximum shortening velocity is likely to represent a compensatory mechanism to maintain power output in the presence of impaired force production. However, it should be noted that these mechanisms appear only to be partially effective in limiting weakness, as evidenced by the prominent symptomatology these patients present with.

Lastly, consideration should be given to the effects of muscle disuse/inactivity on the contractile properties of muscle fibres. As discussed in the preceding chapters, immobilisation has been shown to affect force production, shortening velocity, power output and Ca^{2+} sensitivity.[74,90,111,112] It is important to note, however, that there is a significant difference in the extent of disuse/inactivity between participants in the current

study (mobile, but decreased activity due to weakness and fatigue) and those in the referenced studies (complete immobilisation, bed rest or chronic spinal cord injury). It is therefore not clear whether the findings of the current study could, even partially, be explained by disuse/inactivity. In order to investigate this further, the effect of exercise on contractile properties of muscle fibres from IIM patients will need to be investigated while controlling for the effects of co-interventions, e.g. corticosteroid therapy.

6.2 Future directions

The present study was designed to investigate whether the contractile properties of muscle fibres from individuals with IIMs are altered, and the results of the study could form the basis for future research into possible explanations for the changes in contractile properties. The main finding of the study, impairment in maximum force production, requires further investigation of the underlying mechanisms, in particular the role of inflammatory cytokines such as TNF-α. A study assessing the correlation between TNF receptor (TNFR) expression and contractile properties of muscle fibres from individuals with IIMs would be well suited to investigate this issue, as well as confirm (or refute) the findings of this study. Ideally, muscle tissue should be obtained from patients on at least two occasions: before and after treatment with corticosteroids. As corticosteroids have been shown to inhibit secretion of TNF-α by monocytes and decrease serum levels of soluble TNFR1 and TNFR2, the response to this treatment may prove to be a valuable clue.[54,55] In addition, quantification of myosin concentration may be appropriate, as a linear correlation with force production has been shown in aging and immobilisation.[74] The possibility that inflammation may lead to a decrease in myosin concentration has not

been investigated, and is worth pursuing, although the confounding influence of inactivity due to the disease may prove problematic when interpreting the results.

The impaired force production by muscle fibres from IIM cases shown here, as well as in other muscle disorders,[18-20] is assumed to be a direct consequence of the disease process. However, although a less likely explanation, the possibility that the impaired force production serves to protect the fibre from irreversible damage should perhaps also be considered. A similar situation may be noted in muscle dystrophies, where fibres that are dystrophin deficient lack structural integrity, compared to normal muscle fibres.[155] Since maximal contraction exposes the fibre to substantial shearing forces, limiting force production may therefore serve to protect the structural integrity of the fibre. Although the structural integrity of muscle fibres has not been investigated in IIMs, a personal observation of this investigator is that fibres from IIM cases are substantially more fragile than those from healthy controls. This observation should be considered in future research.

A number of additional findings from the present study are worthy of further investigation. Firstly, as discussed in Chapter 2, the higher proportion of type II fibres (which are capable of higher force and power production) in IIM cases may be a compensatory mechanism. Future studies should first seek to confirm or refute this finding in a larger sample of newly diagnosed, treatment-naïve cases. Secondly, a striking finding is the significantly higher maximum shortening velocities, and consequently power output, of both fibre types in IIMs. Although higher shortening velocities could be explained as a compensatory mechanism in an attempt to preserve power output, the response appears to be excessive and “overcompensating”, resulting in higher power output compared to

healthy controls. This observation raises the question whether the altered maximum shortening velocity is indeed a compensatory mechanism, or rather an unintended consequence of the disease process mediated by, for example, increased passive tension due to collagen deposition or titin stiffening (see Chapter 4). In order to investigate the mechanisms underlying the observed changes in shortening velocity further, passive force measurements can be performed on permeabilised muscle fibres. If passive tension is indeed found to be abnormal, X-ray diffraction studies and quantification of collagen content may be able to identify titin stiffening or increased collagen content (or both) as causative. In addition to passive tension, an increase in actin sliding velocity over myosin should be considered as a mechanism by which maximum shortening velocity can be increased. The *in vitro* motility assay is able to assess this, and should be included in future research.

6.3 Concluding remarks

As is evident from the above, the scope for research into the mechanism(s) by which weakness develops in IIMs is extensive. Ideally, such research should include enough patients to investigate the above issues, not only in IIMs as a group, but also in each of the different disease entities separately, for reasons already alluded to. However, in view of the rarity of these disorders, this may prove difficult. In addition, the laboratory techniques utilised, e.g. permeabilised single fibre studies, are time-consuming and technically demanding, and availability is limited to a few centres with special interest in these techniques.

However, despite the relatively low impact on society due to their rarity, the impact of these disorders on the individual is immense, and they deserve to be investigated with the same urgency as other disorders. Furthermore, unravelling mechanisms of disease such as these, not only has the potential to improve the lives of those affected by the disease, but inevitably, also reveal scientific principles with broader implications to both sickness and health.

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9 APPENDIX A

Notes & Ackowledgements

The methods described here are from Bottinelli, Betto, Schiaffino & Reggiani, 1994; Bottinelli, Schiaffino & Reggiani, 1991; Pansarasa et al., 2008.

Technical expertise and advice has been provided by Matthew Borkowski (Aurora Scientific Inc.) and Dr. Niels Ørtenblad (University of Southern Denmark).

Calculations were performed using the pCa calculator program of Dweck et al. 2006

CHEMICALS

Name	Company	Cat #	Mr	Stock
ATP-$Na_2 \cdot xH_2O$	Sigma	A-2383	551.1	0.1 M
$CaCl_2$	Sigma	C-2661	110.98	0.1 M
Caffeine	Sigma	C-0750	194.19	0.1 M
Creatine kinase	Sigma	C-3755		315U/mg
Creatine phosphate-$Na_2 \cdot 4H_2O$	Roche/Sigma	621722/P-6915	327.2	0.5 M
DTT	Boehringer		154.3	0.1 M
EGTA	Sigma	E-4378	380.4	0.1 M
Imidazole, pKa = 6.95 at 25 °C	Sigma	I-0125	68.08	1.0 M
KCl	Sigma	P9541	74.55	1.0 M
KH_2PO_4	Sigma	P-9791	136.09	0.5 M
KOH	Sigma	P-6310	56.11	5.0 M
Mg-acetate$\cdot 4H_2O$	Sigma	M-5661	214.5	0.5 M
$MgCl_2 \cdot 6H_2O$	Sigma	M-0250	203.3	0.5 M
Propionic acid	Sigma	402907	74.08	13.4 M

CALCULATIONS FOR STOCK SOLUTIONS

0.1 M ATP-Na_2

Weigh off 55.1 mg and make up to 1 ml ddH_2O. Store at -20 °C.

0.1 M $CaCl_2$

Weigh off 0.555 g and make up to 50 ml ddH_2O. Store at -20 °C.

0.5 M Caffeine

Weight off 0.971 g and make up to 10ml ddH_2O. Store in fridge.

0.5 M Creatine phosphate-Na_2

Weigh off 163.6 mg and make up to 1 ml ddH_2O. Store at -20 °C.

0.1 M EGTA

Weigh off 0.951 g and make up to 25 ml ddH_2O. Store at -20 °C.

1.0 M Imidazole

Weigh off 3.4 g and make up to 50 ml ddH_2O. Store at -20 °C.

1.0 M KCl

Weigh off 3.73 g and make up to 50 ml ddH_2O. Store at -20 °C.

0.5 M Mg-acetate

Weigh off 1.073 g and make up to 10 ml ddH_2O. Store at -20 °C.

0.5 M $MgCl_2$

Weigh off 1.0165 g and make up to 10 ml ddH_2O. Store at -20 °C.

5.0 M KOH

Weigh off 14.03 g and make up to 50 ml ddH_2O.

0.5 M KH_2PO_4

Weigh off 3.4 g and make up to 50 ml ddH_2O. Store at -20 °C.

Creatine Kinase (4000 U/ml)

315 U/mg = 1 mg/ml = 315 U/ml → 1 mg / 100 µl = 10 mg/ml = 3150 U/ml

thus $\frac{10\ mg/ml}{x\ mg/ml} = \frac{3150\ U/ml}{4000\ U/ml} = \frac{x\ mg/ml}{3150\ U/ml} = \frac{4000\ U/ml \times 10\ mg/ml}{3150\ U/ml}$

= 12.70 mg in 1 ml ddH_2O – only have 11.11mg, thus 11.11mg / 12.70mg/ml = 875 µl ddH_2O

For every 190 µl activating solution, add 10 µl CK to obtain 200 U/ml.

SOLUTIONS

Skinning solution (pH 7.00)

Stock	[Stock]	For 10 ml	For 50 ml	[Final]
K-Propionate	13.4 M	112 µl	560 µl	150 mM
KH_2PO_4	0.5 M	100 µl	500 µl	5 mM
Mg-acetate	0.5 M	100 µl	500 µl	5 mM
EGTA	0.1 M	500 µl	2.5 ml	5 mM
$CaCl_2$	0.1 M	100 µl	500 µl	1 mM

- Add a few drops ddH_2O so that pH probe would be covered.
- Adjust pH with 5.0 M KOH stirring continuously.

- After adjustment, add the ATP.

Stock	[Stock]	For 10 ml	For 50 ml	[Final]
ATP·Na_2	0.1 M	300 µl	1.5 ml	3 mM

- QS to desired volume, check pH again and adjust accordingly.
- Store at -20 °C in aliquots of 1 ml for 2 months and -87 °C for longer periods.
- Upon first use, dilute 50:50 with glycerol.

EGTA-Imidazole mix

This mix is used when new solutions are made.

Add the following to make a 50 ml mix.

SOLUTION	[STOCK]	VOLUME	[FINAL]
Imidazole	1 M	10 ml	200 mM
EGTA	0.1 M	35 ml	70 mM
dH_2O		5 ml	

Relaxing solution (pH 7.00) : Final ionic strength = 180 mM

NB: Perform pH at 15 °C.

Stock	[Stock]	Volume for 20 ml	[Final]	
EGTA-Imidazole mix		2 ml	7 mM EGTA	
			20 mM Imidazole	
KCl	1.0 M	1521 µl		
$MgCl_2$	0.5 M	220 µl	5.5 mM	pMg = 3.00
$CaCl_2$	0.1 M	2.91 µl	0.02 mM	pCa = 9.00

- Add a few drops dH_2O so that pH probe would be covered.
- Adjust pH with 5.0 M KOH stirring continuously.

- After adjustment, add the ATP and P-Creatine.

Stock	[Stock]	Volume for 20 ml	[Final]	
$ATP{\cdot}Na_2$	0.1 M	1017 µl	5.08 mM	pMgATP = 2.4
P-Creatine	0.5 M	580 µl	14.5 mM	

- Recheck pH and adjust accordingly.
- **Bring final volume to 20 ml with dH_2O.**
- Store at -20 °C in aliquots of 1 ml for 2 months and -87 °C for longer periods.

Pre-activating solution (pH 7.00) : Final ionic strength = 180 mM

NB: Perform pH at 15 °C.

Stock	[Stock]	Volume for 20 ml	[Final]	
EGTA	0.1 M	100 µl	0.5 mM	
Imidazole	1.0 M	400 µl	20 mM	
KCl	1.0 M	2033 µl		
$MgCl_2$	0.5 M	213 µl	5.33 mM	pMg = 3.00

- Add a few drops dH_2O so that pH probe would be covered.
- Adjust pH with 5.0 M KOH stirring continuously.

- After adjustment, add the ATP and P-Creatine

Stock	[Stock]	Volume for 20 ml	[Final]	
$ATP{\cdot}Na_2$	0.1 M	1.029 ml	5.14 mM	pMgATP = 4.00
P-Creatine	0.5 M	580 µl	14.5 mM	

- Recheck pH and adjust accordingly.
- **Bring final volume to 19 ml with dH_2O, NOT 20 ml.**
- Store at -20 °C in aliquots of 1 ml for 2 months and -87 °C for longer periods.
- When preparing the working reagent, add 10 µl CK solution to every 190 µl pre-activating solution.

Activating solution (pH 7.00) : Final ionic strength = 180 mM

NB: Perform pH at 15 °C.

Stock	[Stock]	Volume for 20 ml	[Final]	
EGTA-Imidazole mix		2 ml	7 mM EGTA	
			20 mM Imidazole	
KCl	1.0 M	1240 µl		
$MgCl_2$	0.5 M	213 µl	5.33 mM	pMg = 3.00
$CaCl_2$	0.1 M	1406 µl	7.032 mM	pCa = 4.50

- Add a few drops dH_2O so that pH probe would be covered.
- Adjust pH with 5.0 M KOH stirring continuously.

- After adjustment, add the ATP and P-Creatine

Stock	[Stock]	Volume for 20 ml	[Final]	
$ATP{\cdot}Na_2$	0.1 M	1035 µl	2.66 mM	pMgATP = 2.41
P-Creatine	0.5 M	580 µl	14.5 mM	

- Recheck pH and adjust accordingly.
- **Bring final volume to 19 ml with dH_2O, NOT 20 ml.**
- Store at -20 °C in aliquots of 1 ml for 2 months and -87 °C for longer periods.
- When preparing the working reagent, add 10 µl CK solution to every 190 µl activating solution.

Varying pCa solutions : Final ionic strength = 180 mM

NB: Perform pH at 15 °C.

Stock	[Stock]	pCa 6.8 µl	pCa 6.5 µl	pCa 6.2 µl	pCa 6.0 µl	pCa 5.8 µl	pCa 5.4 µl	pCa 5.1 µl	pCa 4.9 µl	pCa 4.7 µl	Total required ml
EGTA-Imidazole	Mix	1000	1000	1000	1000	1000	1000	1000	1000	1000	9
KCl	1 M	725	704	680	666	653	636	629	626	623	5.9
$MgCl_2$	0.5 M	109	109	108	107	107	107	106	106	106	1.0
$CaCl_2$	0.1 M	182	289	409	483	546	630	666	681	693	4.6

- Add a few drops ddH_2O so that pH probe would be covered.
- Adjust pH with 5.0 M KOH stirring continuously.
- After adjustment, add the ATP and P-Creatine.

	[Stock]	pCa 6.8	pCa 6.5	pCa 6.2	pCa 6.0	pCa 5.8	pCa 5.4	pCa 5.1	pCa 4.9	pCa 4.7	
$ATP{\cdot}Na_2$	0.1 M	509	509	509	509	509	510	511	512	514	4.6
P-Creatine	0.5 M	290	290	290	290	290	290	290	290	290	2.6

- Recheck pH and adjust accordingly.
- **Bring final volume to 9.5 ml each with ddH_2O, NOT 10 ml.**
- When preparing the working reagent, add 10 µl CK solution to every 190 µl relaxing solution.
- Store at -20 °C in aliquots of 1 ml for 2 months and -87 °C for longer periods.